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SUPPLEMENT

August 1957



# CLINICAL CHEMISTRY

*Journal of the American Association of Clinical Chemists*

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# CLINICAL CHEMISTRY

*Journal of the American Association of Clinical Chemists*

**Scope:** CLINICAL CHEMISTRY is devoted to the publication of original and review articles on the application of the science of chemistry to the better understanding of the functioning of the human organism in health and disease.

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Symposia of the  
INTERNATIONAL CONGRESS  
of  
CLINICAL CHEMISTRY

title

September 9-14, 1956, New York

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# CLINICAL CHEMISTRY

*Journal of the American Association of Clinical Chemists*

VOL. 3, NO. 4

AUGUST 1957 (PART 2) SUPPLEMENT

## SPECIAL SUPPLEMENT ISSUE

*Papers Presented as Symposia at the International Congress of  
Clinical Chemistry*

Preface *Harry Sobotka and Harold D. Appleton* 285

John P. Peters *Donald D. Van Slyke* 287

### BLOOD ELECTROLYTES

Aspects of Recent Work on Electrolyte Metabolism  
*C. P. Stewart* 294

The Contribution of Hemoglobin to Acid-Base Equilibrium of  
the Blood in Health and Disease *Rodolfo Margaria* 306

The Role of Magnesium in the Body Fluids  
*J. Russell Elkinton* 319

Electrolyte Disturbances in Acute Uremia  
*Jean Hamburger* 332

Blood Electrolytes under the Influence of Cortical Hormones  
*R. Neher* 344

### PORPHYRINS

The Properties, Estimation Methods, Hematologic Features,  
and Some Other More General Aspects of Different Ab-  
normal Human Hemoglobins *T. H. J. Huisman* 371

(Continued)

Some Aspects of Bile Pigment Metabolism	<i>Rudi Schmid</i>	394
---	--------------------	-----

#### STANDARDIZATION IN CLINICAL CHEMISTRY

Standardization in Clinical Chemistry	<i>I. D. P. Wootton</i>	401
Ultramicro Methods and Standardization of Equipment	<i>M. C. Sanz</i>	406
Standardization in Clinical Chemistry	<i>Marcel Guillot</i>	420
Standardization of Methods in Clinical Chemistry	<i>David Seligson</i>	425

#### ENZYMES

Experiments of Chronic Diabetic Symptoms Caused by Xanthurenic Acid, an Abnormal Metabolite of Tryptophan	<i>Yahito Kotake</i>	432
The Significance of Some B-Complex Vitamins in Clinical Chemistry	<i>Noris Siliprandi</i>	447
Les Acides du Cycle Tricarboxylique en Chimie Clinique (English summary)	<i>Jo Nordmann and Roger Nordmann</i>	462
Enzymes in Clinical Biochemistry	<i>E. J. King</i>	507

#### PROTEINS

Mucoprotein Estimation in Clinical Chemistry	<i>Noel F. MacLagan</i>	548
Mucoproteins in Clinical Chemistry	<i>Z. Stary</i>	557
Significance of Lipoproteins in Clinical Chemistry	<i>J. C. M. Verschure</i>	577

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Members of the International Congress of Clinical Chemistry, 1956		592
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*Symposia of the*  
International Congress  
of  
Clinical Chemistry



## Preface

THE INTERNATIONAL CONGRESS OF CLINICAL CHEMISTRY which was held in New York, September 9-14, 1956, was the first of its kind in the western hemisphere. More than 700 clinical chemists, representing 34 countries, attended and exchanged their ideas and experiences. An alphabetical list of the members will be found at the end of this volume. Among the 150 communications that were presented, twenty reviews were grouped in five symposia, whose central themes were (1) Blood Electrolytes, (2) Porphyrins, (3) Standardization in Clinical Chemistry, (4) Enzymes, and (5) Proteins. It is eighteen of these reviews that comprise the present book. Unfortunately, circumstances have prevented us from publishing in this Supplement the paper "Fluid Compartments and the Excretion of Electrolytes," by Bertil Josephson of the Central Clinical Laboratory of St. Eriks Hospital, Stockholm, Sweden. This paper is being prepared for publication and will appear in a later issue of this volume of CLINICAL CHEMISTRY.

Abstracts of all the communications and descriptions of the many scientific exhibits appeared in CLINICAL CHEMISTRY, August and December 1956 issues [2: 225-295; and 383-393]. They contain papers on serum proteins in hepatic disease, on instrumentation, on enzymatic sugar analysis, on electrophoresis, on various enzymes in the circulation, and on other subjects of timely interest too numerous to list.

The success of the Congress and these lasting records of its work testify to the development of clinical chemistry as a separate discipline, both within the general field of chemistry and amongst the sciences ancillary to medical practice. This trend enabled the Scientific Program Committee to select the subjects for review without undue overlapping with general biochemistry on one hand, or with serology, hematology, etc. on the other hand. Clinical Chemistry seems to represent not so much a bud of biochemistry, but rather a synthesis of analytical chemistry, biochemistry, enzymology, inorganic chemistry, and physical chemistry, in their application to analytic problems in clinical medicine. This situation begins to be acknowledged by the introduction of graduate curricula for the degree of Doctor of Philosophy in Clinical Chemistry in some American universities.

New clinical observations and chemical discoveries alternately widen the

field of clinical chemistry by adding new demands and by answering them with new solutions. The present collection of articles illustrates the development of some branches of clinical chemistry, which are in particularly active progress. It forms a permanent record of an important phase of clinical chemistry.

HARRY SOBOTKA

For the Scientific Program Committee

HAROLD APPLETON

For the Board of Editors



# JOHN P. PETERS

Donald D. Van Slyke

**T**HROUGH FORTY YEARS OF CREATIVE ACTIVITY Peters' work in the study of disease has been characterized by accent on function, and his functional studies have been largely based on chemistry. Clinical chemistry can fairly claim him; he has played a leading part in forging the alliance between chemistry and clinical medicine.

The originality, vigor, breadth of outlook, independence, and intellectual integrity that marked Peters' earliest work continued undiminished to his last recent publication. His contributions to the pathologic physiology of the circulatory, respiratory, excretory, and endocrine systems, of the metabolism of proteins, fats, carbohydrates, electrolytes, and water, have been integrated into the science of medicine. Every medical student, whether he knows it or not, is a student of Peters.

He was a man of many parts. The versatility of his mind was as fascinating as the breadth of his interests and sympathies. His devotion to medicine covered its social as well as its scientific expanse. He had facility in languages, classical and modern, and resultant skill in expression that is not too common among us. Playing the music of the great composers was one of his relaxations.

At work or play his coordination was perfect. In college he captained Yale at the rugged game of water polo. In the laboratory his technic was flawless. He could draw with a precision that was beautiful. Some of the illustrations in McCollum's *Pathology* were done by Peters as a student, and he provided many of those in *Quantitative Clinical Chemistry*.

And withal he was a charming host, friend, and comrade. He was a man magnificent in natural gifts, splendid in uncompromising idealism, inspiring in leadership, generous without limit in the outpouring of his vitality to his science, patients, family, friends, pupils, and country. Through the advances he contributed and the inspiration that he radiated he ranks as one of the great in medicine and in clinical chemistry.

Peters took the M.D. degree at Physicians and Surgeons Medical School, now

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From the Brookhaven National Laboratory, Brookhaven, N. Y.

Columbia College of Medicine, in 1913, and began almost immediately his research activities during his internship and residency at Presbyterian Hospital. His first independent contribution "Carbon Dioxide Acidosis, the Cause of Cardiac Dyspnea," in 1917, shows the qualities that throughout have distinguished his work: interest in the functional effects of disease, study of them by both observation and experiment, and keen, critical evaluation of the results. He showed in this early paper that the  $\text{CO}_2$  tension in the blood is increased by interference with excretion in the lungs, and that the dyspnea results from the attempt to overcome the retention by greater ventilation.

From May, 1917, to March, 1919, Peters was in France with the Presbyterian Hospital Unit. The routine demands of war service did not prevent his carrying out with Alexander Raymond Stevens an important study of war nephritis.

Returning to New York, he spent the academic year 1919-20 on the faculty of Cornell Medical School, and with David Barr made contributions to the acid-base balance to which we will refer again.

During the year 1920-21 Peters joined the group with which I was associated at the Hospital of the Rockefeller Institute and took part in initiating a series of studies on blood and electrolyte equilibria. Peters' skill in the laboratory and his keen critical sense made most valuable his part in this early work. The personal association and collaboration begun at this time continued through the subsequent decades.

Peters left the Rockefeller Institute to return to his collegiate alma mater, Yale, where, in the Department of Medicine, he was leader in studies of metabolic medicine up to the day of his final illness, less than a year ago. His work at Yale was carried out with collaborators who contributed to the results and profited from association with an inspired chief. The group included the chemists, Eisenman, Man, and Hald, and the internists, Winkler, Bulger, Lavites, Wakeman, Danowski, Elkinton, and others. Most of those who had the inspiration of working with Peters have continued active to the present date.

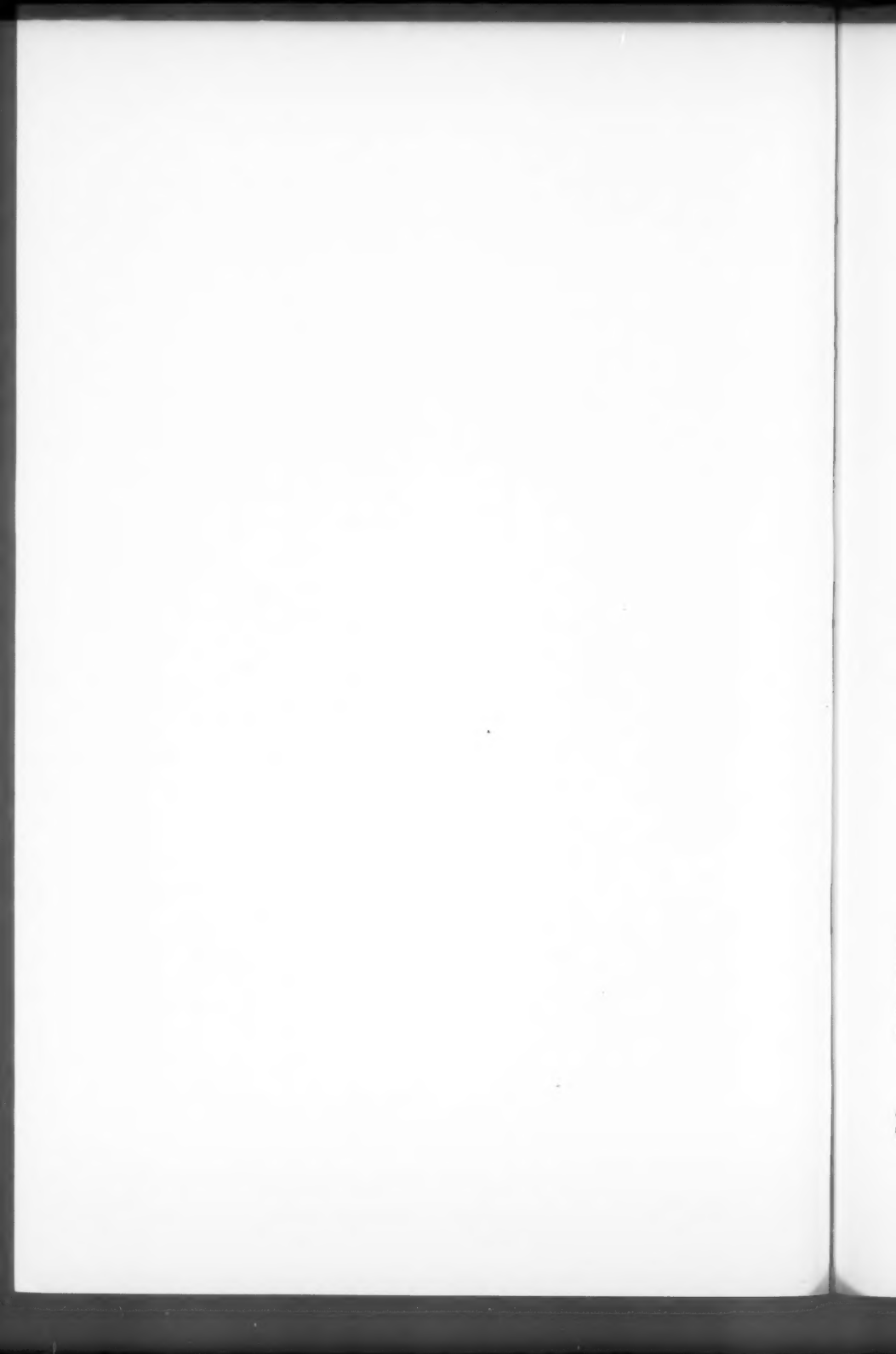
Besides his research contributions, the eminence that Peters early attained led to his being invited to write many reviews, which he provided with the same keen discrimination employed in his experimental work. These reviews included the books, *Quantitative Clinical Chemistry*, published in 1931, and *Body Water*, in 1935. It was my privilege to associate with him in writing *Quantitative Clinical Chemistry*, and I must acknowledge that if it had not been for his example of untiring energy in that work, my own part of it would have never been finished.

With regard to *Body Water*, it may be said that although published twenty-one years ago, few papers in the field at present appear without reference to this integration of the subject that we owe to Peters.

Peters' work has covered intensively many fields. The following is a very incomplete summary of his contributions to some of them.



John P. Peters, M.D., 1887-1955  
Scientist, Teacher, Philosopher



### ELECTROLYTES AND THE ACID-BASE BALANCE

Peters' first contribution, his 1917 paper on  $\text{CO}_2$  acidosis in cardiac dyspnea, which we have mentioned, was in this field.

In the same year Peters and Geyelin showed that epinephrine administration caused a decrease in alkali reserve accompanied by an increase in blood lactic acid. This appears to be the first report showing the effect of adrenalin in the production of lactic acid.

In 1920, with David Barr of Cornell University Medical School, Peters published a series of papers on blood  $\text{CO}_2$  absorption curves and alkali reserve which typify his method of planned attack on a problem. The normal limits were first carefully defined together with physiologic variations (including effects of exercise in this case). Then the conditions in cardiac failure, emphysema, and nephritis were studied with a keen evaluation of the factors which caused the alterations observed.

At Yale studies of blood electrolytes and acid-base balance carried on with Hald, Winkler, Danowski, Elkinton, and others, added through a series of significant papers to knowledge of physiologic and pathologic relations of these factors. These studies showed, with many other findings, that potassium transfer in and out of the red cells depends on metabolic activity of the cells; that organic phosphorus from the red cells is lost in diabetic acidosis; that diabetic acidosis can involve loss of sodium chloride and potassium as well as bicarbonate, and that the dehydration and salt loss are attributable not to polyuria of hyperglycemia, but to other conditions of the acidosis. Eventually the studies led to the influences of nervous and hormonal origin and showed that salt depletion in diseases of the central nervous system may be important.

### CARBOHYDRATE METABOLISM AND DIABETES

Peters' studies of diabetes were not limited to the acid-base balance but covered practically all the metabolic factors that could be studied with clinical material. As mentioned above, he showed that dehydration and loss of sodium chloride and depletion of serum potassium could occur in diabetic acidosis. In 1925 a paper showing that in diabetic coma the kidneys excrete a dilute urine provided one of the early pointers indicating the occurrence of the shock kidney as a complication of diabetic coma. The effect of insulin was studied not only on the blood sugar but also on the blood electrolytes, the acid-base balance, and the water balance of the body. The series of papers from Peters' clinic contributed to an important degree to present methods in the treatment of diabetes.

### LIPIDS

Studies of blood lipids and fat metabolism were begun with Evelyn Man about 1943 and continued through to papers published during the past year. These studies, like those of the acid-base balance, began with investigations of

the normal physiologic conditions and thorough testing of methods. In two papers with Man, Albrink, and Glenn, appearing in 1955, the transport of lipids in the chyle of surgical patients was studied under conditions that permitted systematic collection of chyle. These studies showed in human subjects that the lipid uptake from the intestine into the chyle is chiefly in the form of neutral fats. Studies of the serum fats showed that the ability of the serum proteins to dissolve neutral fats as lipoprotein complexes is limited to about 500 mg. of neutral fats per 100 ml. of serum, and that when the concentration of neutral fats exceeds this limit lactescence results. Lipemic conditions studied included diabetes, nephrosis, alcoholic fatty liver, essential hyperlipemia, and pancreatitis. The results of these studies form an important addition to the physiology as well as to the pathology of fat metabolism.

### PLASMA PROTEINS AND THEIR RELATION TO HYDRATION AND EDEMA

These studies began to appear in 1925 in collaboration with Eisenman and Bulger and were initiated, as was the usual case with Peters' work, by a careful study of the range of normal conditions. The concurrence of plasma albumin deficit with edema in various conditions, particularly nephrosis, had been noticed by others and emphasized by Epstein as a probable cause-and-effect phenomenon. Peters' work added experimental evidence by producing edema in animals as a result of plasma albumin deficit experimentally caused by protein malnutrition. In this work Peters was associated with Lafayette Mendel, the early master of nutrition studies on rats. The clinical studies showed that in conditions of various origin that lowered the plasma albumin concentration below 3 Gm. per 100 cc., edema was almost invariable. Peters' studies added to those of Epstein and Govaerts in showing the importance of the osmotic suction of the plasma albumin in maintaining water equilibrium in the body and in the prevention of edema. The interest in water physiology culminated in 1935 in the publication of Peters' well-known book, *Body Water*, which is still the standard work on the subject. His studies of factors affecting hydration continued and resulted in publication, with Elkinton, Danowski, and Winkler, of a series of papers on the relation of dehydration to shock. This work provided some of the most important evidence in showing that a state of shock is caused by decrease in circulating blood volume and that treatment of the acute condition calls for infusion of fluids containing not only salts but also plasma proteins or other colloids which can provide similar osmotic suction to retain water in the circulating plasma.

### THYROID AND IODINE PHYSIOLOGY

Peters was one of the first clinicians to recognize the importance of the determination of plasma thyroxine in the diagnosis of thyroid hyper- and hypofunction and to make prolonged and thorough studies of plasma changes in guiding therapy in these conditions. Again his studies began with tests of

analytical technic and determination of normal values in a paper published in 1940 with Riggs and Man. These studies showed, among other things, that the protein-bound iodine concentration in serum could be decreased in conditions of albumin deficit, such as nephrosis, unaccompanied by thyroid malfunction, and that in pregnancy the PBI might be as high as 10 or 11 gamma per 100 cc. instead of the normal 6 to 7. During these studies the analytical technic was improved by a paper with Kidd and Man in 1951 in which it was shown that thyroxine could be quantitatively extracted from serum with butanol, and that the determination of iodine in the extract provided a more reliable estimate of plasma thyroxine than was yielded by iodine determination performed on the plasma protein precipitate.

### NITROGEN METABOLISM

Through a large part of his clinical studies the nitrogen metabolism of Peters' patients was followed by observation of the nitrogen balance. Results in detail are too many to discuss in the time available. One finding in a paper published in 1946 seems to deserve especial mention. It was entirely new and had therapeutic implications of importance. It showed that the content of free alpha amino acids in surgical patients was likely to fall much below normal during several days after operation, and that the deficit was proportional to the severity of the operation. The reactions of nitrogen metabolism to injury and to acute and chronic disease are broadly studied throughout Peters' clinical reports.

### SOCIAL RESPONSIBILITIES OF MEDICINE

Peters' devotion to the scientific aspects of medicine did not dull his sense of social responsibility as a physician nor his sensitivity as a humanitarian. During the last twenty years of his life he spoke and published repeatedly on the social responsibilities of medicine. He felt keenly the responsibility of the medical profession to the public and at the same time realized the necessity for caution in altering the conditions for medical care that have evolved on the basis of past experience. His stand may be summarized in two quotations: "My social philosophy, I presume, would be termed radical, though I prefer to believe myself merely intelligently open-minded. . . . [But] a sweeping program suddenly imposed on the country would create confusion if not chaos. Thoughtful investigation and experimentation promise more than grandiose projects born of emotional preconceptions."

# Aspects of Recent Work on Electrolyte Metabolism

*C. P. Stewart, D.Sc., Ph.D.*

THE ENORMOUS OUTPUT OF PAPERS dealing with various aspects of electrolyte metabolism reflects both the importance of this subject and the growing possibility of studying it in detail which is provided by the flame photometer and by the use of radioactive isotopes. The interest in electrolytes seems to be autocatalytic; each advance, either because of its own intrinsic importance or because of the relationship it reveals with other parts of clinical chemistry and of medicine, becomes the stimulus to a host of further researches in, often, the most diverse directions. To review adequately this vast field would require not one paper, or even a symposium, but a considerable series of lectures, and this is still true if one narrows the field to "blood electrolytes" (which, after all, merely mirror, and that imperfectly, the interchanges summed up in the term "metabolism"). I have therefore ventured, under the prescribed general heading, to select certain topics which appear to be of rather general interest at the moment as well as being of especial interest to me. Even so, I can only treat them rather sketchily and hope that I may give an acceptable general outline of some recent advances, with minimal distortion and without undue oversimplification.

## TRENDS IN CLINICAL CHEMISTRY

Advances in analytic technic have tended, in recent years, to focus attention on the alkali metals and on the principal anions of the blood plasma to such an extent that the term "blood electrolytes" is coming to connote these almost to the exclusion of the other inorganic ions present in much smaller amounts. However that may be, it is of chloride and bicarbonate of potassium and sodium that I wish chiefly to speak.

The study of the blood—and urine—electrolytes has proceeded in a way which seems to me to exemplify very well the role and the values of clinical

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From the Department of Clinical Chemistry, University of Edinburgh, Clinical Laboratory, Royal Infirmary, Edinburgh, Scotland.



chemistry. It has shown a variety of abnormalities, some quite unexpected a few years ago, and, by providing analytic methods which are reasonably accurate when applied to small amounts of material, has made it possible for the clinician to obtain objective data as an aid to diagnosis or prognosis.

There has been a tendency to regard this provision and use of diagnostic tools as being the chief if not the only object of clinical chemistry. No one would seek to deny the importance of developing new or improved analytic methods which may provide additional or better chemical aids to diagnosis, but to my mind an even greater aim of clinical chemistry is to elucidate the biochemical lesions of disease and so lead to such an understanding of the disease processes that often the very occurrence of the disease may be prevented, and that when it does occur the diagnosis may be facilitated, the treatment put upon a rational basis and the prognosis readily ascertained. In achieving this aim the use of the chemical tests necessary in the study and, at an early stage, essential as a part of the diagnostic procedure may become less and less required in clinical routine except as providing objective evidence for record purposes or vivid examples for teaching. An example of this is afforded by potassium depletion, the existence of which nowadays is diagnosed largely on clinical evidence—although not so long ago the determination of the plasma potassium concentration was regarded as an essential step (insufficient though it is now recognized to be). The present position has been reached only because the intensive application of clinical chemistry has led to a sufficient understanding of the conditions in which potassium depletion may occur, of the clinical signs it produces, and of the relation between the plasma concentration of potassium on the one hand and the quantity and distribution of potassium in the body on the other hand.

This differentiation of routine diagnostic work from the investigation of disease—e.g., the distinction between investigating whether or not a given patient has impaired renal efficiency and studying the etiology of nephritis—does not involve any conflict of interests. The data acquired in routine work may well be utilized in a planned investigation justifying the name of research. But that clinical chemists are indeed deeply interested and engaged in the study of disease is shown by the considerable proportion of papers dealing with subjects of this kind and the relatively few concerned solely with the valuable, though less exciting elaboration of new or improved diagnostic tests.

During the past few years the interest of many clinical chemists seems to have been focused, so far as blood electrolytes are concerned, on potassium (and particularly upon potassium depletion) and on the interrelations between electrolytes and endocrine activity. It is of certain parts of this work that I propose to speak now, taking, however, the broad view that blood or plasma concentrations being the resultant of intake, internal interchange, and output, their consideration must involve electrolyte "metabolism" in general. Indeed one of the most striking results of the investigations of recent years has

been the realization that far-reaching abnormalities of electrolyte distribution and "metabolism" may often occur without more than small alterations in the plasma concentrations.

### ACID-BASE BALANCE

In many abnormalities of electrolyte metabolism, including those which are at present being studied intensively, there occurs, either as an essential or an incidental part of the general disturbance, an alteration in what is still commonly termed the acid-base balance but which is much better described as the acid-base ratio or the hydrogen ion control. No single term, however, is ideal for what is usually meant by the term acid-base balance which includes both the ratio of acid concentration to base concentration—i.e., the hydrogen ion concentration—and the buffering capacity, which in practice depends not on this ratio but on the total concentration of effective base. Many clinicians have considerable difficulty in understanding the nature of acidosis and alkalosis, their causation, their compensation or repair by natural means, and their rational treatment. Even biochemists, who certainly ought to know better, have made such understanding more difficult for themselves than it need be. For much of the trouble is undoubtedly due to the almost universal use of the term "acid" to signify anions and the term "base" to signify cations—an erroneous usage which has led to endless confusion. Without imputing blame to anyone, it seems well worth while to call attention to this, and to suggest that by using a terminology more consonant with modern chemical theory, many misconceptions, inconsistencies, and paradoxes disappear. Partial attempts in this direction have recently been made in a number of monographs and papers, but it may be suggested that the time is now ripe for more drastic reform, as has been urged by Welt (1955), by Owen (1955), and by Owen and Robson (1956).

In terms of the theory proposed by Lowry (1924) and by Brönsted (1926), an acid is a donor of hydrogen ions, and a base is an acceptor of hydrogen ions. An acid is thus essentially an undissociated molecule or complex ion which on dissociation yields a hydrogen ion (which, in water, may then combine with the solvent) and an anion; a base is generally but not always an anion. In this, cations other than hydrogen ions have no part as such; they are merely required to maintain electrical neutrality and their role is consequently passive. It is to be noted that on this view the cation  $\text{NH}_4^+$  is important not because it is a cation but because it is actually an acid. On the other hand, the molecule  $\text{NH}_3$  is a base because it can accept  $\text{H}^+$  and become the acid  $\text{NH}_4^+$ . Some substances (e.g.,  $\text{H}_2\text{PO}_4^-$ , protein) can act as either acids or bases, being truly amphoteric and able either to donate or accept hydrogen ions—usually according to the pH of the solution. In the following list the acids are arranged in order of decreasing strength and the bases, consequently, in order of increasing strength; protein, however, may not always be in the position assigned

to it here, some proteins being stronger acids than carbonic acid, and the actual acid under physiologic conditions being probably protein ion rather than an undissociated molecule.

<i>Acid</i>	<i>Base</i>
HCl	$H^+ + Cl^-$
$H_2PO_4^-$	$H^+ + HPO_4^{2-}$
$H_2CO_3$	$H^+ + HCO_3^-$
HPr	$H^+ + Pr^-$
$NH_4^+$	$H^+ + NH_3$

In this group  $Cl^-$  is so weak as to be physiologically useless as a base. The predominant bases of the body fluids are bicarbonate ions, monohydrogen phosphate ions, and protein ions in those fluids which contain protein in considerable concentration. A base, in presence of its acid and hydrogen ion, forms an "acid-base system" which acts as a buffer system whose effectiveness depends upon such factors as the degree of dissociation of the acid, the relative concentrations of acid and base, etc. In a mixed solution all the acid-base systems must be in mutual equilibrium.

It is clear that disturbance of the hydrogen ion regulation (or acid-base balance) can exist only when there is abnormal accumulation or loss of acid or base. Abnormality in the concentration of the neutral cations sodium, potassium, etc. may be an accompanying or even a causative phenomenon—or a sequel—but cannot itself constitute either acidosis or alkalosis. Equally, abnormality of the concentration of those anions which are too weak to function as bases (e.g.,  $Cl^-$ ) may be present but is not in itself a *part* of the acid-base imbalance.

On this basis, the acidosis of severe chronic diarrhea is not due to the loss of sodium or potassium (though that occurs) but to the loss of a fluid relatively rich in the strong base  $HCO_3^-$ .

In a recent publication it was stated that "the part played by the kidney in the production of . . . extracellular alkalosis was therefore to reabsorb too much sodium and too little chloride" (Fitzgerald and Fourman, 1955); had the term "bicarbonate" been used instead of "sodium" the situation would have been correctly described and it could have been added that this might have involved secondarily the reabsorption of more sodium than usual—if the amounts of  $HCO_3^-$  and  $Cl^-$  together exceeded the original total.

Another published statement is that "although metabolic alkalosis may be produced by relative excess of sodium, it usually results from relative deficit of chloride (Darrow and Pratt, 1950). It is true that the concentrations of these ions, which are neither acids nor effective bases, may and often do alter in alkalosis. However, the metabolic alkalosis of vomiting, for instance, is not due to chloride loss but to the loss of hydrochloric acid, which produces in the body a relative excess of base. Again, acidosis results from ingestion of am-

monium chloride because the ammonium ions though certainly showing but little tendency to dissociate, do so slightly, and when the  $\text{NH}_3$  so formed is continually removed as urea, glutamine, etc., the simultaneously formed  $\text{H}^+$  cumulatively combines with some of the strong base (i.e., buffer anion) and so reduces the concentration of base. It would be easy to multiply examples but in the present circumstances little would be gained by doing so, and enough has been said to show how the use of the Brönsted-Lowry concept of acids and bases leads to a clearer appreciation of the disturbances in the acid-base ratio and avoids the confusion and errors which inevitably appear when acidosis and alkalosis are described in terms of abnormalities in sodium, potassium, and chloride.

This is not to deny the importance even in relation to  $\text{H}^+$  control (i.e., acid-base ratio), of abnormalities in concentration of metallic cations and of anions which, like  $\text{Cl}^-$  are not effective bases at least under physiologic conditions. Indeed they may contribute most importantly to the clinical picture and may even be an essential part of it. They may at times have a causative role. Primary loss of sodium, for example, does not *per se* produce acidosis, since the sodium ion is neither acid nor base. But loss of cation necessarily means concurrent and equal loss of anion (since electrical neutrality must be maintained in all circumstances), and this latter may involve a loss of bicarbonate (base) and so produce an acidosis. Similarly, impairment of renal function may sometimes affect, primarily, certain cations (e.g., potassium), and although this altered excretion of an ion which is neither acid nor base can have no direct effect on the acid-base ratio, again the accompanying change in anion (base) excretion may alter the ratio as in certain forms of potassium deficiency.

Since the "long-term" regulation of the acid-base ratio in the face of disturbance involves renal excretion of extra acid or base, and since this extra excretion must involve altered excretion of covering ions, there must inevitably be a tendency for alterations in the concentrations of cations and/or chloride to occur as part of the compensatory and restorative mechanisms in abnormalities of the acid-base ratio. The changes are undoubtedly complex but much of the complexity can be resolved and a clearer and more readily explainable picture both of root causes and of the sequence of events can be obtained if we discard the old and erroneous terminology in favor of the newer concepts of acids and bases.

### METABOLIC CHANGES AFTER INJURY

Work extending over many years and summarized in several recent reviews (e.g., Symposium in *Brit. Med. Bull.*, 1954; Stewart, 1955) and monographs (e.g., Moore and Ball, 1950; Wilkinson, 1955) has established the existence of a "normal" metabolic response to injury. This response includes an increased output of urea, potassium, and phosphate, together with a decreased output of sodium and water. These disturbances, however, do not appear to coincide

either in onset or duration. Nor are they equally influenced by administration of the appropriate metabolites—the negative balance of nitrogen can be reduced by giving protein during the immediate postoperative period, though most observers agree that it cannot be completely abolished; the magnitude of a sodium “retention” may even be increased by administering more sodium chloride, but is said to be reduced by massive transfusion of blood; it has been reported that even when these abnormalities are largely modified, the postoperative potassium loss continues (Flear and Clarke, 1955). It is noteworthy that although an internal water shift has been recognized, with some increase in the total extracellular fluid volume, the plasma concentrations of potassium, sodium, and chloride show only small and inconstant changes (Wilkinson *et al.*, 1951).

The general pattern of these metabolic changes has suggested that they may be caused by endocrine disturbances involving in particular the adrenal cortex. It has, indeed, been shown in several laboratories, using a variety of nonspecific analytic methods, that the urinary output of steroids of adrenocortical origin increases during the immediate postoperative period. An increase in the blood concentration of “17-hydroxycorticoids” has also been demonstrated and evidence adduced to show that it represents a real increase in secretory activity and not merely a decreased rate of metabolic breakdown. Moreover, both Llauro (1955) and Zimmermann (1955) have reported increased urinary output of aldosterone during the period immediately following surgery, and the latter author concludes that this increased hormonal activity adequately explains the positive sodium balance observed postoperatively. There is thus little doubt that an increase in the production of adrenal hormones—including aldosterone—is a normal sequel to surgical trauma. But important though this is, it seems clear that the metabolic response in terms of protein breakdown and electrolyte disturbances is not proportional to the increased secretory activity of the adrenal cortex and may even be, at least partly, independent of it. This is, indeed, essentially the conclusion reached by Ingle, Ward, and Kuizenga (1947) on the basis of observations on adrenalectomized rats, and it has only recently been tested in man. Some of the published reports of the metabolic response to adrenalectomy are difficult to interpret because of variations in the doses of cortisone, but in the case described by Robson, *et al.*, (1955) and in several of those reported by Mason (1955) this difficulty does not arise. Mason found that when patients were given a constant daily dose of cortisone (60 or 80 mg. I.M.) beginning some 2–3 days before operation, adrenalectomy (plus oophorectomy) was followed by the normal metabolic response. In my own laboratory we found a similarly “normal” metabolic response in a patient who underwent removal of the second adrenal gland whilst being maintained on a daily dose of 200 mg. cortisone orally; in this case the urinary output of acid-stable formaldehydogenic steroids was measured and found to be constant for the 3 days preceding and

the 5 days following the operation. Both groups have also observed the normal metabolic response after hypophysectomy, the patients being given either corticotrophin or cortisone in constant dosage. It is evident that the metabolic and electrolyte responses to surgical operation do not depend entirely on increased general adrenocortical secretion; they may well do so partially but not enough information is yet available for quantitative conclusions to be drawn. However, the responses reported by Mason and by the Edinburgh group (combined with other, as yet unpublished, data from the latter) are certainly not very much smaller than those given by patients with intact adrenals undergoing surgery of comparable severity and, of course, showing the usual increased output of adrenal hormones. It can be said that the adrenal cortex plays a "permissive" role, since there is evidence (e.g., Ingle *et al.*, 1947) that the increased protein breakdown which normally follows injury does not occur when there is neither secretion nor administration of adrenocortical hormones, but this is a concept which certainly needs further examination.

In any case it must be accepted that surgical interference is ordinarily accompanied or followed by increased secretion of adrenal hormones—and indeed by other changes in endocrine activity—and it is reasonable to suppose that the hormones will produce their usual physiologic changes, adding to, modifying, or being modified by the metabolic disturbances due to other (possibly direct tissue) phenomena. It is perhaps apposite here that the urinary excretion and probably the actual secretion rate of aldosterone can apparently be modified by changes in the body water—it is said to be decreased when water is retained, and increased by diuresis (Muller, Riondel, and Mach, 1956)—such as appear to occur as a sequel to injury.

If there is still uncertainty about the mechanism involved in the production of the electrolyte and other disturbances which occur as a normal concomitant of surgery, there is equal uncertainty about two matters which affect the clinical biochemist in his routine work—what analyses he should advise as a means of following the postoperation course of events and what measures he should advise in order to abolish the disturbances. According to one view, the "normal" disturbances constitute a physiologic response to injury and should not be actively combated; only if the clinical condition of the patient shows that the usual reversal of the changes is not occurring do active measures become necessary. This point of view is ably argued by Wilkinson (1955). Others consider that it is desirable from the beginning to restore the electrolyte and protein balance as nearly as possible to the preoperation state, and they claim that this regimen confers such benefits as shortened convalescence (e.g., Elman, 1955). Both parties agree, of course, that any untoward event, any deterioration in the patient's clinical state, demands full investigation and active treatment.

So far as electrolytes and their accompanying solvent are concerned, the

main dangers appear to be (1) excessive retention of water, sodium, and chloride, with the development of clinical edema (often but not always the result of excessive saline administration); (2) excessive or prolonged potassium loss, often with resultant hypokalemia, accompanied by accumulation of intracellular sodium and/or loss of intracellular water (usually the result, if renal function is adequate, of failure to supply potassium when the initial cellular loss of potassium is beginning to be reversed or of inadequate water intake or continued loss of gastrointestinal secretions); (3) abnormalities of the acid-base ratio.

To guard against or to overcome excessive sodium retention, overinfusion with saline is to be avoided (saline per rectum may also favor sodium retention) and the use of hypotonic saline solution with glucose or of "mixed electrolyte" and glucose solutions is usually to be preferred, with careful adjustment of dosage to the calculated losses. Frequent determination of plasma potassium is probably a desirable precaution, especially when this ion is being given intravenously.

The administration of potassium salts at the time when the excessive post-operative loss is in full swing (i.e., during the 2 or 3 days immediately after operation) seems undesirable but should probably be routine when the urinary output is again falling, if by that time the intake in food is still inadequate. But infusion is required only when the oral route is not available. In this, as in other forms of potassium depletion, diagnosis of the condition must be made largely on clinical grounds; objective chemical evidence is frequently difficult to obtain and the results of chemical analysis of blood and urine are not easy to interpret. Indeed this applies also to sodium retention and it is by no means invariably true that the one is indicated by a low plasma potassium and the other by a high plasma sodium concentration, as has recently been discussed by Scribner and Burnell (1956). Even where these abnormalities of plasma composition do in fact exist, they cannot necessarily be taken at face value. It is a commonplace that potassium depletion can exist in the presence of a normal plasma concentration if there has been excessive loss from the cells; conversely a low plasma potassium concentration is consistent with absence of depletion, if the extracellular fluid volume is markedly increased. Clearly then the chemical investigation of a possible potassium depletion or sodium retention involves not only determination of these ions in the plasma but also measurement of the body water compartments, and probably of the plasma bicarbonate as well, since either metabolic alkalosis or acidosis may be present. Usually, however, such detailed investigation is required only to provide objective evidence for retrospective consideration of the case; the diagnosis for practical purposes can generally be made on clinical grounds.

But where objective evidence is likely to be needed, attention may be called to the value of serial urine analyses, especially when they can be related to measured intakes. Assuming the patient to have been normal so far as electro-



lyte concentration and balance are concerned before the operation, it is evident that a potassium depletion of serious proportions can develop only if the expected temporary loss during the first 1-3 postoperative days continues, either because intake is inadequate or because potassium-losing conditions remain operative. Since the intake is usually known in such conditions, serial urine analyses can often indicate impending danger. The value of urine analysis is probably smaller in the case of excessive sodium retention, partly because the "normal" period of retention is longer than the period of potassium loss, and partly because the intake of sodium is often less accurately known. However, if one single determination had to be chosen to supplement clinical observation in diagnosing and following excessive sodium retention, it would probably be that of extracellular fluid volume.

### POTASSIUM DEPLETION

Since Thorn and his associates (Thorn, Koepf, and Clinton, 1944) described a condition of renal failure in which there was excessive loss of sodium and chloride associated with a normal plasma pH, a tubular failure to reabsorb sodium which was not overcome by administration of D.O.C.A., "salt-losing nephritis" has become recognized as a rather unusual but quite real syndrome. Apart from this pseudo-Addison's disease, however, sodium depletion associated with renal disease is by no means uncommon and may result from a variety of causes such as excessive loss by vomiting, diarrhea, etc., or insufficient intake of salt in relation to water. True salt-losing nephritis merits close investigation, for it may well assist in giving a clearer picture than we yet possess of the relation between renal function and hormones (especially those of the adrenal cortex).

Concentrations of plasma potassium below the accepted normal range have also been stated to occur with moderate frequency in renal disease (Hamburger *et al.*, 1954). This, of course, does not necessarily point to the existence of an actual potassium depletion in these cases, and, in any case, small losses of potassium seem to produce no important clinical signs (Black and Milne, 1952). However, the symptoms reported by Fourman (1954) as accompanying experimental depletion of body potassium to the extent of about 30 per cent are often encountered in patients with renal disease, and in some cases at least the renal insufficiency may be the cause of marked potassium depletion—in other words, there may well be a "potassium-losing" renal disease.

Obviously the clinical and biochemical signs need careful evaluation, for a marked potassium depletion may be due to overproduction of aldosterone, and indeed this has been claimed as the true explanation of a case originally described as one in which the potassium wastage was primarily due to renal disease. It is possible, of course, that disturbance of aldosterone production plays a part in all cases, for potassium depletion itself has been stated to decrease aldosterone excretion (Luetscher and Curtis, 1955; Singer and Stack-



Dunne, 1955) so that "normal" values for aldosterone in such conditions may really be indicative of oversecretion (Garrod, Simpson, and Tait, 1956)—i.e., of adrenal tumor and primary adrenal, rather than primary renal, causation. Until the methods of aldosterone assay are much more sensitive—and practicable—than they are at present, the important distinction between true potassium-losing nephritis in which the primary cause is renal, and similar conditions in which an adrenal tumor is the real agent must be made on clinical and chemical grounds.

Tentatively, it seems that it may be worth while to consider the possibility of there being three possible types. In the first, with evidence of adrenal tumor, evidence of normal or nearly normal renal function, the potassium depletion may be ascribed to the adrenal dysfunction as the primary cause; this is the primary aldosteronism of Conn (1955), and quite probably the cases reported by Cope and Milne (1955) fall into this group. The other two types both present evidence of renal disease. In one of these, exemplified by the case which Mahler and Stanbury (1956) investigated in great detail, no evidence of adrenal hyperactivity could be found by urine and blood examination or physically at laparotomy, and this type may be regarded as true potassium-losing renal disease, a condition involving, presumably, an abnormal renal response to a normal production of aldosterone. In the third group, of which the case of Chalmers *et al.* (1956) seems to be an instance, renal disease is certainly present, but careful search also reveals the existence of an adrenal tumor; here we are left quite uncertain as to whether the adrenal lesion follows or results from the renal damage, whether the opposite is the proper sequence and the renal disease is caused by the adrenal tumor or by the hypokalaemia resulting therefrom (Schwartz and Relman, 1953; Merrill, 1956), or whether the coexistence of the two lesions is purely fortuitous. Admittedly, of course, there is the possibility that the second and third of these groups are really only one and that the apparent absence of the adrenal factor in one is merely due to the insufficiency of the means of detecting it. Further, there is clearly the possibility that in all three groups the adrenal and the renal factors are both present in varying degree. But these considerations do not make the classification useless as a working hypothesis; they merely emphasize the desirability of further detailed investigation of potassium depletion and they serve to point out certain of the directions which these investigations must take.

It is of practical importance to distinguish those cases in which the adrenal factor is fundamentally at fault, for in primary aldosteronism surgery offers the possibility of real relief. In their detailed study of one case which the available evidence suggests to be one of true potassium depletion of renal origin, Mahler and Stanbury (1956) give some help in the problem of making a differential diagnosis. (They also promise a review of the whole problem of renal disease and potassium deficiency, to be published in the near future.)

These authors point out that in their patient there was definite evidence of renal disease—a chronic pyelonephritis diagnosed after examination of biopsy material as well as on clinical and biochemical grounds—with a markedly reduced capacity for urine acidification persisting long after the potassium deficiency had been corrected. This is important, for many of the clinical signs (anorexia, nocturia, etc.) and some of the chemical signs (albuminuria) are described also in aldosteronism. Further, in the patient of Mahler and Stanbury the potassium clearance varied only from 10 to 20 ml./min. (the simultaneously measured clearance of inulin and creatinine was 62 ml./min.) over the range of plasma potassium concentration from 2.5 mEq./l. when depletion was present to 6.3 mEq./l. after complete correction; in primary aldosteronism (Evans and Milne, 1954; Earle *et al.*, 1951) and in renal excretory failure (Platt, 1950) the potassium clearance may approach or even exceed the glomerular filtration rate. The effect of the renal dysfunction was to keep the capacity for excreting potassium within a relatively narrow range whatever the plasma concentration of potassium might be; in primary aldosteronism, on the other hand, the higher the plasma potassium level the greater the rate of excretion.

Still another important point of differentiation is that, whereas in potassium depletion of adrenal origin correction by potassium dosage is difficult, Mahler and Stanbury found that administration of potassium salts readily made good the potassium deficit of their patient and that careful control by determination of plasma potassium concentration was necessary to prevent overdosage and hyperkalemia. There was indeed some evidence, which may be highly important, that during the period of potassium replenishment the plasma concentration readily rose above the normal, whilst tissue depletion still remained. Some of the metabolic abnormalities found in this patient—e.g., a reduced power of conserving sodium and a marked phosphaturia associated with a low plasma phosphate concentration—appeared to be the effects of the potassium deficiency rather than of the primary renal lesion, and were therefore of little diagnostic importance. A valuable suggestion made by Baxter (1956) is that in primary aldosteronism sodium deprivation results in a rapid disappearance of sodium from the urine, whereas in “potassium-losing nephritis,” secondary aldosteronism, such deprivation may actually uncover an obligatory sodium loss.

Clearly the certain differentiation of true potassium-losing renal disease from potassium depletion of adrenal origin is not easy and a reliable method of aldosterone assay with careful study of its interpretation is urgently needed. But even this may not solve completely the problem of rapid diagnosis, if only because of the complexity of the biologic and chemical methods available, and careful investigation of tubular function, of potassium excretion in relation to plasma concentration of potassium and of the response to potassium administration or sodium deprivation on the lines suggested by Mahler and Stan-

bury and by Bartter may well be required. It is only by such detailed studies that advances will be made in this interesting and important field.

### REFERENCES

1. Bartter, F. C., *Metabolism* **5**, 369 (1956).
2. Black, D. A. K., and Milne, M. D., *Clin. Sci.* **11**, 397 (1952).
3. British Med. Bull. **10**, No. 1 (1954).
4. Brönsted, J. N., *J. Phys. Chem.* **30**, 777 (1926).
5. Chalmers, J. M., Fitzgerald, M. G., James, A. H., and Scarborough, H., *Lancet* **270**, 127 (1956).
6. Conn, J. W., *J. Lab. Clin. Med.* **45**, 661 (1955).
7. Cope, C. L., and Milne, M. D., *Brit. Med. J.* **i**, 969 (1955).
8. Darrow, D. C., and Pratt, E. L., *J. Amer. Med. Assoc.* **143**, 365 (1950).
9. Earle, D. P., Sherry, S., Eichna, L. W., and Conan, N. J., *Amer. J. Med.* **11**, 283 (1951).
10. Elman, R., *Proc. 16<sup>th</sup> Congrès International de Chirurgie*, Copenhagen, (1955).
11. Evans, B. M., and Milne, M. D., *Brit. Med. J.* **ii**, 1067 (1954).
12. Flear, C. T. G., and Clarke, R., *Clin. Sci.* **14**, 575 (1955).
13. Fitzgerald, M. G., and Fourman, P., *Lancet* **269**, 848 (1955).
14. Fourman, P., *Clin. Sci.* **13**, 93 (1954).
15. Garrod, O., Simpson, S. A., and Tait, J. F., *Lancet* **270**, 860 (1956).
16. Hamburger, J., Crosnier, J., Funck-Brentano, J. L., Rapin, C., and Masson, J., *Physiopathologie du Potassium*. Paris, p. 115 (1954).
17. Ingle, D. J., Ward, E. O., and Kuizenga, M. H., *Amer. J. Physiol.* **149**, 510 (1947).
18. Llaurodo, J. G., *Lancet* **268**, 1295 (1955).
19. Lowry, T. M., *Trans. Faraday Soc.* **20**, 13 (1924).
20. Luetscher, J. A. Jun., and Curtis, R. H., *Fed. Proc.* **14**, 746 (1955).
21. Mahler, R. F., and Stanbury, S. W., *Quart. J. Med.* **25**, 21 (1956).
22. Mason, A. S., *Lancet* **269**, 632 (1955).
23. Merrill, J. P., *Metabolism* **5**, 419 (1956).
24. Moore, F. D., and Ball, M. R., *The Metabolic Response to Surgery*, Springfield, Ill., Thomas (1952).
25. Muller, A. F., Riandel, A. M., and Mach, R. S., *Lancet* **270**, 831 (1956).
26. Owen, J. A., *J. Roy. Inst. Chem.*, Nov., 565 (1955).
27. Owen, J. A., and Robson, J. S., *Scot. Med. J.* **1**, 294 (1956).
28. Platt, R., *Clin. Sci.* **9**, 367 (1950).
29. Robson, J. S., Horn, D. B., Dudley, H. A., and Stewart, C. P., *Lancet* **269**, 325 (1955).
30. Schwartz, W. B., and Reiman, A. S., *J. Clin. Invest.* **32**, 258 (1953).
31. Scribner, B. H., and Burnell, J. M., *Metabolism* **5**, 468 (1956).
32. Singer, B., and Stack-Dunne, M. J., *Endocrinology* **12**, 130 (1955).
33. Stewart, C. P., *Proc. 16<sup>th</sup> Congrès International de Chirurgie*, Copenhagen (1955).
34. Thorn, G. W., Koepf, G. F., and Clinton, M., Jr., *New England J. Med.* **231**, 76 (1944).
35. Welt, L. G., *Clinical Disorders of Hydration and Acid-Base Equilibrium*, London, Churchill (1955).
36. Wilkinson, A. W., *Body Fluids in Surgery*, Edinburgh and London, Livingstone (1955).
37. Wilkinson, A. W., Billing, B. H., Nagy, G., and Stewart, C. P., *Lancet* **i**, 315 (1951).
38. Zimmermann, B., *41st Clin. Congr. Amer. Coll. Surg.* (1955).

# The Contribution of Hemoglobin to Acid-Base Equilibrium of the Blood in Health and Disease

*Rodolfo Margaria*

**T**HE CONTRIBUTION OF HEMOGLOBIN to the acid-base equilibrium of the blood does not need to be emphasized: it is an old problem, to which classical work of outstanding figures in physiology, such as Bohr, Haldane, Barcroft, Henderson, and Van Slyke, is connected.

It is well known that in this respect hemoglobin does not only behave as other proteins, i.e. as a partially ionized weak acid with strong buffer properties; its peculiarity is that its acidic properties change with oxygenation. This phenomenon, well known as the "Bohr effect" can be described either as a shift to the right of the  $O_2$  dissociation curve for blood by adding  $CO_2$ , or to a displacement to lower values of the  $CO_2$  dissociation curve for blood by adding  $O_2$ .

The shift to the right of the  $O_2$  dissociation curve by  $CO_2$  was attributed to a lesser ionization of a peculiar acidic group of Hb due to the necessarily involved increase in acidity (8, 11, 23, 24). Margaria and Green (1933), however, observed that the dissociation curve of Hb in presence of  $NaHCO_3CO_2$  is sensibly more displaced to the right than the curve of a similar Hb solution in absence of  $NaHCO_3CO_2$  at the same pH and at the same ionic strength. the electrolyte being replaced by NaCl.

This effect clearly could not be attributable to a change of dissociation of Hb, since no change of pH was involved, and the authors attributed it to the formation of a  $CO_2Hb$  complex, having less affinity for  $O_2$  than simple Hb. The formation of this  $CO_2Hb$  compound was made evident also by earlier work by Margaria (1931) in which this was detected osmotically, and by still earlier work by Henriques (1928). This discovery showed the need for a revision of our knowledge concerning the acidic properties of Hb, since it was not known how  $CO_2Hb$  would behave as an acid.

Some indications could be found, however, in the previous literature: particularly significant was the finding by the Van Slyke school that titration

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curves of sodium hemoglobinate solutions in the pH range 6 to 8 were apparently the same whether HCl or CO<sub>2</sub> had been used as an acid (8, 10, 23). This was interpreted by Margaria and Green (1933), assuming that the acidic properties of CO<sub>2</sub>Hb are higher than those of Hb, to mean that exactly the same acidity would be achieved as by the addition of an equivalent amount of CO<sub>2</sub> to the solution.

The binding of CO<sub>2</sub> with Hb was assumed by Henriques (1928-1929) to be of a carbamate nature. The dissociation of the new acidic group resulting from a carbamino reaction was studied by Faurholt (1925) on simpler substances, such as glycine and many other aminoacids or simple amines, and it is of such a magnitude as to give the reason for the higher acidity of the complex. The nature of this binding, however, which was only given as an hypothesis by Henriques, was discussed by Margaria and Green (1933), by Roughton (1935, 1943-1944), Stadie and O'Brien (1937), and by Margaria (1952) and only in the last years it was elucidated partially.

The amount of CO<sub>2</sub> bound with hemoglobin was estimated with two different methods that gave rather different results. Margaria and Green (14) used the formula

$$(\text{CO}_2\text{Hb}) = (\text{CO}_2 \text{ tot.}) - (\text{CO}_2 \text{ diss.}) - (\text{HCO}_3^-) - (\text{CO}=\text{O})$$

where CO<sub>2</sub> total and CO<sub>2</sub> diss. (dissolved) were measured in the conventional way, the first with the Van Slyke manometric apparatus and the second by calculation from CO<sub>2</sub> analysis on the air in equilibrium with the solution; the (HCO<sub>3</sub><sup>-</sup>) and (CO=O) were calculated from Henderson's formula or from the second dissociation of the carbonic acid equation, the pH being measured, and the pK calculated as from Hastings and Sendroy (1925), the ionic strength of the solution being known.

The second is a chemical method (Siegfried 1909, Faurholt 1925), and it is based on the removal of all HCO<sub>3</sub><sup>-</sup> and CO=O ions by precipitation with Ba<sup>++</sup> in a very alkaline medium; the carbamate CO<sub>2</sub> remains in solution, and it can then be determined chemically, or with the Van Slyke technic (Ferguson and Roughton, 1935).

The discrepancy between the two methods consisted mainly in the fact that the first method gave appreciable amounts of CO<sub>2</sub> bound with Hb at pH less than 7.0, a point lower than the isoelectric point of Hb. The chemical method does not show the existence of any CO<sub>2</sub> bound with Hb at pH of 7.0 or less. It was in fact mainly on the basis of a high value of CO<sub>2</sub>Hb at pH < 7 that Margaria (1952) based doubts on the carbamino mechanism of such a binding, the carbamino binding taking place with most aminoacids and other simple amines only at pH of 9 to 11 (Faurholt, 1925); furthermore, other proteins such as serum albumin or casein did not bind any appreciable amount of CO<sub>2</sub>, carbamino or not, at pH < 7.5-8.0 (Giustina, Milla, Margaria, 1953).

The carbamino binding with Hb had never been demonstrated with cer-

tainty, but only postulated, as the most likely of the possible chemical reactions of a protein with  $\text{CO}_2$ .

Such a discrepancy was pointed out long ago by Roughton (1935, 1943-1944) who called Y-bound  $\text{CO}_2$  the fraction that could not, and X-bound  $\text{CO}_2$  the fraction that could be accounted for as a carbamino compound. This hypothesis however of the existence of two mechanisms for the binding of  $\text{CO}_2$  with Hb could only be accepted with reluctance, being based on results of different authors working under different experimental conditions.

Contrary to my personal expectations, however, such a hypothesis was confirmed by work done in my laboratory in Milan. Giustina, Milla, and Margaria (1952, 1953) showed it in fact in a series of papers concerned both with the kinetic of the formation of  $\text{CO}_2\text{Hb}$  and with determinations of bound  $\text{CO}_2$  at equilibrium, carried at the same time and on the same solution with the chemical method and with Margaria and Green's procedure, on both oxygenated and reduced hemoglobin, on a pH range from 6 to 12. Experiments run on simple aminoacids and on other proteins (Milla, Giustina, Margaria, 1953; Giustina, Milla, Margaria, 1953) showed that the chemical (Faurholt's) method of measuring  $\text{CO}_2\text{Hb}$  is in effect a rather specific method for carbamino-compound, and only to the  $\text{CO}_2\text{Hb}$  detected with this method the name of carbhemoglobin will be restricted.

This compound starts forming at pH of about 7.0, increases up to a maximum at pH 10, decreasing progressively down to zero at pH 12 ab.: this behavior is very similar to the binding of  $\text{CO}_2$  with glycine to form a carbamino-glycine (Fig. 1).

At the physiologic range of pH, however, only part of  $\text{CO}_2\text{Hb}$  is carbhemoglobin: the other fraction starts forming at pH 7.4 and is found in higher proportion at lower pH (Figs. 2 and 3). Its chemical nature is not known (Milla, Margaria, 1954).

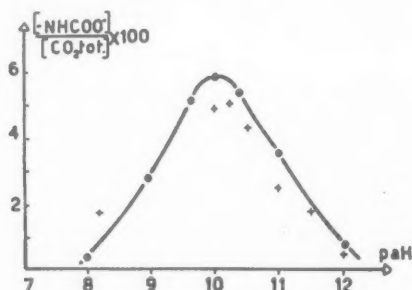


Fig. 1. Concentration of  $\text{CO}_2$  as  $\text{CO}_2\text{Hb}$  (+) in a 3 mM/l. Hb solution or as carbamino glycine (·) in a 10 mM/l. glycine solution, as a function of pH, at 20°. In both cases ( $\text{CO}_2$  tot.) = 100 mM/l. (From Milla, Giustina, and Margaria, 1953.)

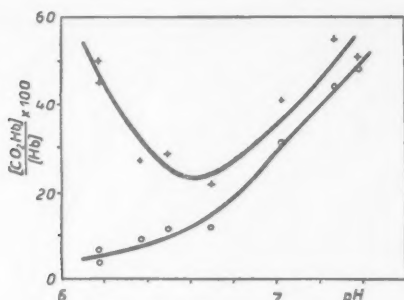


Fig. 2.  $\text{CO}_2$  bound with reduced hemoglobin as a function of  $\text{pH}$  at  $37^\circ$ .  $+$  = total (found with Margaria and Green's method.)

$o$  = carbamino-bound  $\text{CO}_2$  (Faurholt's method). Similar data are obtained with  $\text{HbO}_2$ , only the  $\text{CO}_2$  bound with hemoglobin is sensibly less. (From Milla, Giustina, and Margaria, 1953).

The presence of these two kinds of  $\text{CO}_2\text{Hb}$  at physiologic  $\text{pH}$  raised the problem of what effect each of them would have on the acidity of Hb and the  $\text{O}_2$  dissociation curve of normal blood.

The effect of oxygenation and of the presence of  $\text{CO}_2$  on the dissociation of the acidic groups of Hb, the buffer value of this protein at different experimental conditions, have all been treated on theoretical and experimental ground mainly by L. J. Henderson (1920) and Van Slyke (1924); their excellent and stimulating work has been the starting platform for all further research (German and Wyman, 1937, and Wyman, 1939-1948) that has been made since in this field.

After the discovery that  $\text{CO}_2$  binds with hemoglobin, and that there are therefore in blood two kinds of this protein capable of binding with  $\text{O}_2$ , i.e., Hb and  $\text{CO}_2\text{Hb}$ , no attempt had been made to investigate quantitatively the different acidic properties of these two substances: even the effect of  $\text{CO}_2$  independent from  $\text{pH}$  on the dissociation curve of Hb for  $\text{O}_2$  was evidenced by Margaria and Green (1933) only at a single  $\text{pH}$  value, 7.37, at which it was shown later that both  $\text{CO}_2\text{Hb}$  compounds are present in appreciable amounts. It is true that the identity of the titration curves of Hb with  $\text{HCl}$  as with  $\text{CO}_2$  (Hastings, Sendroy, Murray, *et al.*, 1925) was rather discouraging in this respect, because it made appear that  $\text{CO}_2$  had the same effect on the  $\text{pH}$  of the solution whether it was simply dissolved in the solution, or combined in the Hb molecule. A revision of the effect of  $\text{CO}_2$  on the  $\text{O}_2$  dissociation curve of Hb solutions, and reciprocally of the effect of  $\text{O}_2$  on the acidic properties of Hb and of its compounds with  $\text{CO}_2$  was felt needed over the whole range of  $\text{pH}$  6 to 8. A possible additional evidence of the two mechanisms of the combination of  $\text{CO}_2$  with Hb could have been given by this analysis.

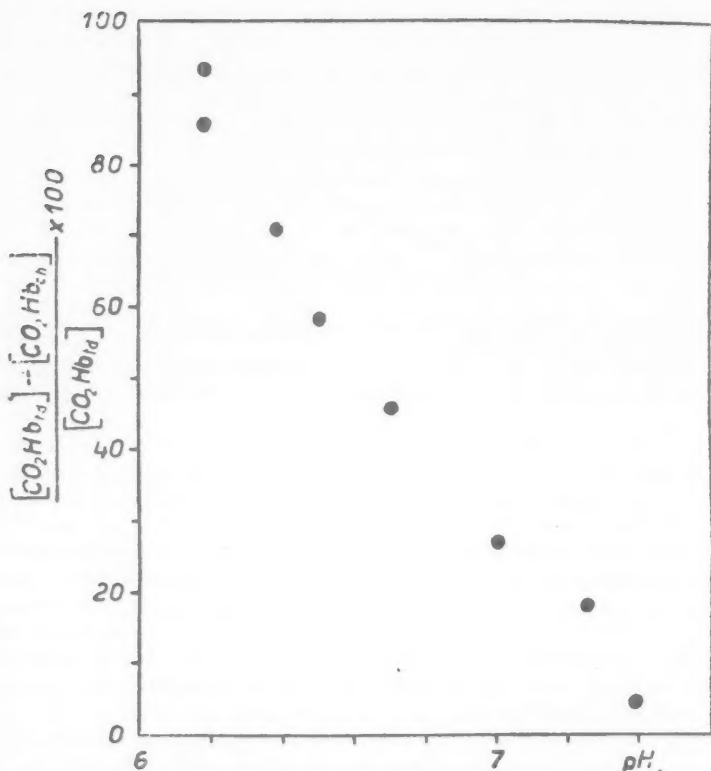


Fig. 3. Percentage of noncarbamino CO<sub>2</sub> of the total CO<sub>2</sub> bound with reduced Hb, as a function of pH. (From Milla, Giustina, and Margaria, 1953.)

A set of dissociation curves for O<sub>2</sub> of the same Hb solution both in NaHCO<sub>3</sub>-CO<sub>2</sub> and in absence of CO<sub>2</sub>, the ionic strength being kept constant, has been worked out experimentally, over the greatest possible range of pH (Margaria and Milla, 1955) (Fig. 4).

The effect due to the presence of CO<sub>2</sub> could be calculated as the difference in the *p*O<sub>2</sub> at a given saturation value, between the data obtained in NaHCO<sub>3</sub>-CO<sub>2</sub> and in CO<sub>2</sub>-free solutions.

In Fig. 5 the *p*O<sub>2</sub> at 50 per cent saturation is plotted as a function of pH both in presence and in absence of CO<sub>2</sub>. Such *p*O<sub>2</sub> values are higher in presence of CO<sub>2</sub> in the whole range of pH used, nor is it possible to detect a difference in



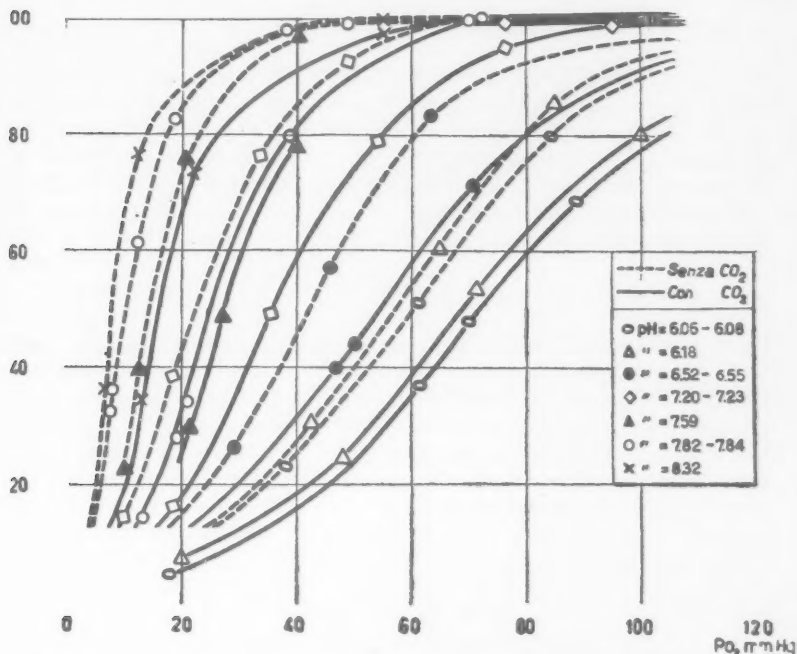


Fig. 4. Oxygen dissociation curves of the same horse Hb preparation in  $\text{NaHCO}_3\text{CO}_2$  (continuous line) or in absence of  $\text{CO}_2$  (dotted line) at pH values as indicated. For all experiments (Hb) = 10 mM/ (on the assumption that its m.w. = 17,000) and ionic strength = 0.1. The different pH values were obtained adding to the original NaHb solutions either  $\text{CO}_2$  or lactic acid (a weak acid was preferred to HCl, to prevent alteration of Hb in solution).

behavior in the range of high pH values where carbamino compounds formed, in relation to lower pH values.

The incline of the curves of Fig. 5, i.e. the differential quotient  $d\text{PO}_2/d\text{pH}$  appears to be very near the same for both curves; this means that a change of  $\text{pO}_2$  at 50 per cent oxygenation would correspond to about the same change in acidity whether the Hb is bound with  $\text{CO}_2$  or not.

But more important than the effect of variation of  $\text{pO}_2$  is the effect of change of oxygenation of Hb on pH,  $d\text{HbO}_2/d\text{pH}$ . This can be calculated by measuring the incline of all the curves of Fig. 4 at 50 per cent oxygenation,  $d\text{HbO}_2/d\text{PO}_2$ , and multiplying them by the incline values of the curve of Fig. 5 at the corresponding pH values.

The  $d\text{HbO}_2/d\text{pH}$  values for Hb at 50 per cent oxygenation and for all pH

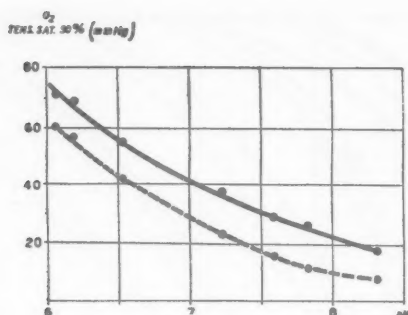


Fig. 5.  $pO_2$  for 50 per cent saturation (data from Fig. 4) are plotted against  $pH$ . Continuous line refers to Hb in presence of  $NaHCO_3CO_2$ , dotted line in presence of NaCl of same ionic strength. (From Margaria and Milla, 1955.)

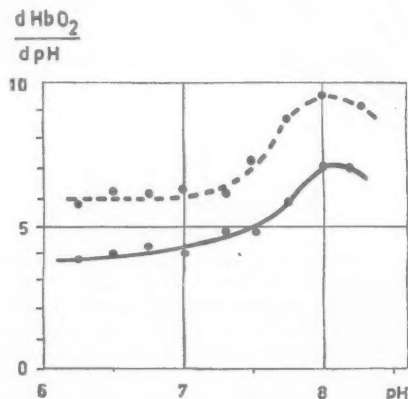


Fig. 6. Acidic effect of oxygenation of hemoglobin in  $NaHCO_3CO_2$  solution (continuous line) and NaCl (dotted line). (From Margaria and Milla, 1955.)

values are given in Fig. 6 for both sets of curves, in presence and in absence of  $CO_2$ .

The two curves run parallel, again not showing any difference in behavior at  $pH$  ab. 7.0, where the  $CO_2$  combination with Hb changes from the carbamino mechanism to the other. The curve for  $CO_2$ , however, is all the way at a lower level than the curve in absence of  $CO_2$ , which shows that a change in oxygenation of  $CO_2Hb$  has a greater effect on the acidic properties of hemoglobin than a change of oxygenation of uncombined Hb. Furthermore, for both Hb and  $CO_2Hb$  the increase in acidity due to oxygenation is maximum at low  $pH$

(6.0); it decreases moderately and steadily up to pH 7.5, to go down to a low minimum at pH 8.0.

The effect of oxygenation on pH depends not only on the change of the acidic properties of hemoglobin, but also on the buffer value of the solution. This is defined by  $dB/dpH$ , calculated on a titration curve of a hemoglobin solution. By dividing the values  $dB/dpH$  by the buffer value referred to the oxygenation of Hb,  $dHbO_2/dpH$ , the value  $dB/dHbO_2$  is obtained: this defines the amount of base (or acid) in mol corresponding to the subtraction (or addition) of 1 mol of  $O_2$ , and the acidifying properties of oxygen are described in terms of addition or subtraction of acid or base.

Titration curves of hemoglobin, oxyhemoglobin, and carboxyhemoglobin solutions are numerous in the literature (Van Slyke, Wu, McLean, 1923; Hastings *et al.*, 1924-1925; German and Wyman, 1937; Wyman, 1939; Cohn, Green and Blanchard, 1937), but particularly interesting are for the present argument those by Hastings, Van Slyke, *et al.* (1924) and of Hastings and Sendroy, *et al.* (1925), in which the titration was made using both HCl and  $CO_2$  as acids. It is well known that these authors could not find any difference using these two acids. This result, though not being necessarily in conflict with the hypothesis of the formation of a  $CO_2Hb$  complex, made this possibility not so probable. Such titration curves, however, were made at a time when the glass electrode for pH measurement was not yet introduced in the practice, and the data obtained could not be so reliable as can be obtained nowadays with modern technics. Furthermore, the titration curve for  $CO_2$  (Hastings, Van Slyke, *et al.*, 1924) was limited in the pH range 6.8 to 7.6.

For these reasons titration curves of hemoglobin were worked out again on the same hemoglobin preparations on which the other experiments were performed. These results are described in summary in Fig. 7. They confirm substantially the previous findings by Hastings, Van Slyke, and their collaborators (1924-1925) in the sense that in the limits of experimental error the titration curve with  $CO_2$  is the same as with HCl.

No indication is given in the titration curve of the existence of a compound of  $CO_2$  with Hb, and this neither at pH >7.0 where a carbamino compound is formed, nor at lower pH, where another compound has been evidenced.

The data  $dB/dHbO_2$  as calculated from Figs. 6 and 7, are given in Fig. 8 as a function of pH for a 50 per cent Hb saturation with  $O_2$ . These data, referred to in the  $CO_2$  experiments, are of the same order of magnitude as the data given by Hastings, Van Slyke, *et al.* (1924). The only difference is that the maximum values are not found at pH 7.4, but are displaced toward the acid side, at pH 7.0. The absolute values (at these two pH's) however are not significantly different.

The values referring to the experiments made in absence of  $CO_2$  are appreciably lower than those obtained in presence of  $CO_2$ , the maximum of  $dB/dHbO_2$  being 0.5 instead of 0.7: these are not to be compared with Hastings

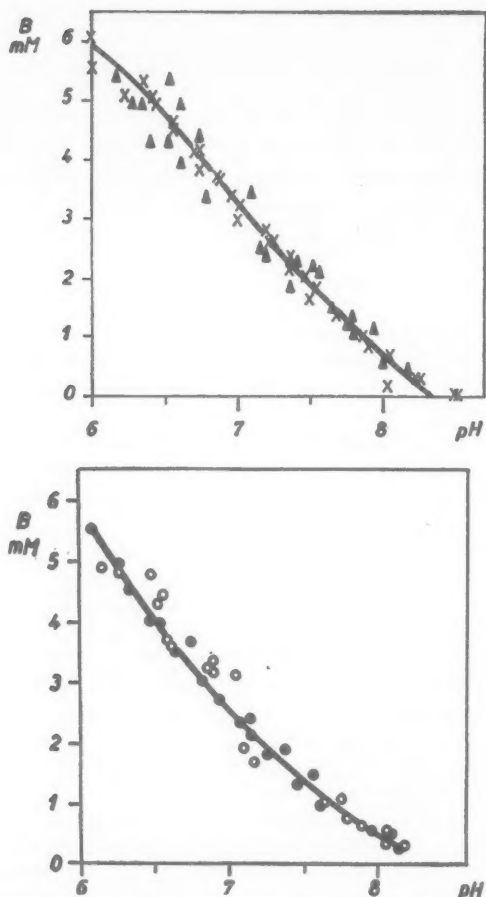


Fig. 7. A, titration curves of NaHb solutions with CO<sub>2</sub> (triangles) and HCl (crosses). B, titration curves of NaHbO<sub>2</sub> solutions with CO<sub>2</sub> (circles) and HCl (dots). The concentration of Hb was 7-10 mM. Ordinate: mM acid added per mM of protein in solution.

and Van Slyke's data (1924), as these last were obtained experimenting on solutions of Hb in presence of NaHCO<sub>3</sub>CO<sub>2</sub>. Data such as those of Fig. 8 can be obtained not only for 50 per cent, but also for all values of saturation of Hb with O<sub>2</sub>; each set of such data  $\frac{dB}{dHbO_2}$  can be plotted as a function of oxygenation at a given pH, as described in Fig. 9 for pH values 7.2, 7.4, and 7.6.

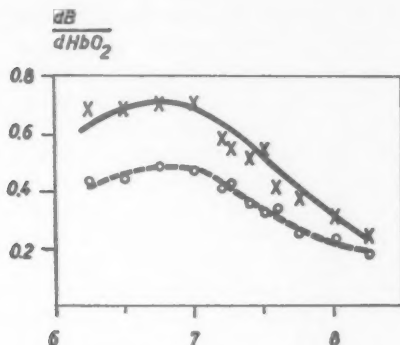


Fig. 8. Acid equivalents of oxygenation of Hb as a function of pH, in presence of  $\text{NaHCO}_3\text{-CO}_2$  (continuous line) or of NaCl (dotted line). (From Margaria and Milla, 1956.)

The data  $\text{dB}/\text{dHbO}_2$  as given by Van Slyke (1924) have been calculated on the difference between the base binding power of Hb relative to  $\text{HbO}_2$ . No effect of a change of the acidic properties of Hb has been measured for intermediate values of oxygenation, and this has been thought to be constant (1923). The data in Fig. 9 show, however, that this is not the case. The base equivalent of  $\text{O}_2$  binding to Hb is lowest at about 60 per cent oxygenation, having a value  $\text{ab.} = 0.5$ , increasing to about 1.0 at saturation with  $\text{O}_2$ . At low oxygenation it increases also up to about 1.0 in absence of  $\text{CO}_2$ , and up to as much as about 2.0 in presence of  $\text{NaHCO}_3\text{CO}_2$ .

It is true that under ordinary resting conditions the base equivalent of  $\text{O}_2$  is 0.7, the same value as given by Hastings, Van Slyke, *et al.* (1924), as it is evidenced in Fig. 9, where the approximate arterial and venous points are indicated. It is shown in this diagram that the first molecules of  $\text{O}_2$  that are being taken up in the lungs by venous blood increase the acidity very little, only about as much as the assumption of 0.5 mol of  $\text{CO}_2$ . Only at the end of the oxygenation process the assumption of oxygen implies the same acidic change as the absorption of an amount  $\text{ab.}$  equivalent of  $\text{CO}_2$ .

The venous blood entering the pulmonary capillary vessels, its characteristics being defined by the point *v* on the diagram of Fig. 9, first loses  $\text{CO}_2$ , as the diffusion of this gas takes place at a very rapid rate, and only later the Hb is oxygenated, the diffusion of  $\text{O}_2$  taking place at a much slower rate. The loss of  $\text{CO}_2$  by the blood causes the point *v* to shift to a lower value on the diagram, toward the broken line representing the acidic properties of Hb in absence of  $\text{HCO}_3\text{-CO}_2$ ; a contribution to a shift in the same direction is given also by higher alkalinity of the blood due to the  $\text{CO}_2$  loss. Only later in the length of the pulmonary capillary, when  $\text{O}_2$  is admitted to the Hb molecule, there will be a shift to the right along the area of the base-binding effect of  $\text{O}_2$

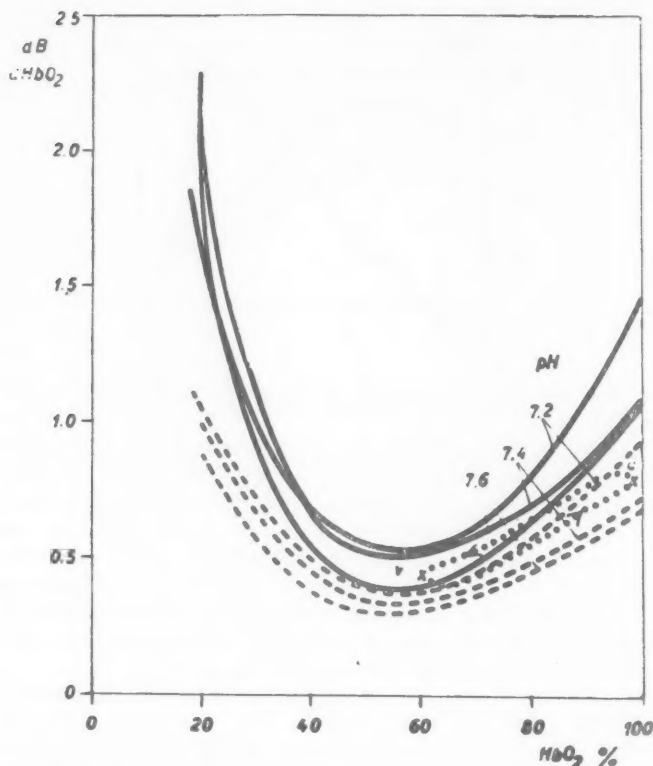


Fig. 9. Acidic equivalents of oxygenation of Hb as a function of  $O_2$  saturations.

The three continuous lines refer to the effect on Hb in presence of  $NaHCO_3CO_2$  at the pH indicated. The three dotted lines refer to analogous experiments in absence of  $CO_2$ .  $v$  = normal venous point (rest conditions).  $\alpha$  = normal arterial point. (From Margaria and Milla, 1956.)

(see Fig. 9). The pH of the medium is so restored toward the original value, the main effect being shown only by the end of the oxygenation process. A reverse mechanism takes place in the tissues.

This process shows a somewhat different behavior if the venous blood coming to the lungs is very much reduced and rich in  $CO_2$ . In this last case the beginning of oxygenation of Hb may imply a strong acidification, corresponding to the absorption of 1 to 2 mols of  $CO_2$  per mol of  $O_2$  absorbed, and this would immediately compensate for the high alkalization due to the  $CO_2$  loss; only

later, toward the 60 per cent oxygenation, the acidification would go to a lower pace, to increase again towards 90-100 per cent oxygenation.

It certainly seems peculiar that such a strong effect that  $\text{CO}_2$  has on the oxygenation of Hb, independently from pH, has no correspondence on the titration curves of Hb and  $\text{HbO}_2$ , as it has since long been observed by Hastings, Van Slyke, *et al.* (1924) and Hastings, Sendroy, *et al.* (1925). This cannot be explained, I think, except assuming that the  $\text{CO}_2$  that combines with Hb, whether it is a carbamino compound at  $\text{pH} > 7.0$ , or another binding at lower pH, retains its acidic properties unaltered, as suggested in 1933 by Margaria and Green. How this can take place for carbamino compounds is well understandable from the physicochemical behavior of these substances as developed by Faurholt (1925). It is much less understood how it may take place at pH values of  $< 7.0$ .

Certainly the existence of a  $\text{CO}_2\text{Hb}$  compound at  $\text{pH} < 7.0$  is somewhat distressing; nevertheless, there cannot be doubt of its existence, unless a more plausible explanation is found for the low  $pK_1$  values for carbonic acid, in such range of pH, found by Van Slyke *et al.* (1925); Stadie and Hawes (1928); Dill (1937), and for the results, calculated on similar experimental basis, obtained by Margaria (1931), Margaria and Green (1933); and Milla, Giustina, and Margaria (1953).

In a normal acid-base condition the value of  $\text{dB}/\text{dHbO}_2$  averages about 0.7, as shown in Fig. 9, in agreement with the value given 30 years ago by Van Slyke, Hastings, *et al.* (1924); it implies that with a R.Q. of 0.7 no difference in pH could be detected between arterial and venous blood.

This statement, however, cannot be taken as a generalization. In physiologic conditions, when a high oxygen desaturation of venous blood is observed, *i.e.* during strenuous muscular exercise, the high value of  $\text{dB}/\text{dHbO}_2$  at low (20 per cent) oxygen saturation is compensated by the small value at saturation 40-80 per cent, and the over-all mean value of such a coefficient is not appreciably changed.

A shift toward higher or lower values, however, can be observed in *acidosis* or *alkalosis*. An increase in acidity tends to increase the values for  $\text{dB}/\text{dHbO}_2$  at high  $\text{O}_2$  saturation, though not influencing appreciably such values at about 50 per cent  $\text{O}_2$  saturation: the average value for the base equivalent of  $\text{O}_2$  bound with Hb is then increased, and at pH 7.2 may be as high as ab. 1.0.

The highest values would of course be observed during *respiratory acidosis*, because both factors—acidosis and  $\text{CO}_2$ —contribute to an increase of the factor  $\text{dB}/\text{dHbO}_2$ . In *metabolic acidosis*, which implies a decrease of  $\text{CO}_2$ , the tendency of  $\text{dB}/\text{dHbO}_2$  would be (a) toward an increase because of the higher acidity, (b) toward a decrease because of less  $\text{CO}_2$  and a lower proportion of the  $\text{CO}_2\text{Hb}$  compound in the blood. Because of the two conflicting directions of the displacement, no great over-all change of this coefficient can be expected either on *metabolic acidosis* or *alkalosis*.

Only in *hypercapnic acidosis* or in *hypocapnic alkalosis* the two components of the  $\text{d}B/\text{dHbO}_2$  shift act in the same direction, and a maximum value will be observed in the first case, a minimum in alkalosis. The description as given indicates that in a  $\text{CO}_2$  acidosis with a decrease of blood pH to ab. 7.2, the acidity of venous blood may not be higher, or it may even be slightly lower, than the acidity of arterial blood, even at high values of R.Q. Inversely the greatest difference between arterial and venous pH would be observed in respiratory alkalosis.

## REFERENCES

1. Cohn, E. Y., Green, A. A., and Blanchard, M. H., *J. Am. Chem. Soc.* **33**, 780 (1937).
2. Dill, D. B., Daly, C., and Forbes, W. H., *J. Biol. Chem.* **117**, 569 (1937).
3. Faurholt, C., *J. Chim. Physiol.* **22**, 1 (1925).
4. Ferguson, Y. K. W., and Roughton, F. J. W., *J. Physiol.* **83**, 68 (1935).
5. German, B., and Wyman, J., *J. Biol. Chem.* **117**, 533 (1937).
6. Giustina, G., Milla, E., and Margaria, R., *Giornale di Biochimica* **11**, 357 (1953); **1**, 357, 475, (1952).
8. Hastings, A. B., Van Slyke, D. D., Neill, J. M., Heidelberger, M., and Harrington, C. R., *J. Biol. Chem.* **60**, 89 (1924).
9. Hastings, A. B., and Sendroy, J., Jr., *J. Biol. Chem.* **65**, 445 (1925).
10. Hastings, A. B., Sendroy, J., Jr., Murray, C. D., and Heidelberger, M., *J. Biol. Chem.* **61**, 317 (1925).
11. Henderson, L. J., *J. Biol. Chem.* **41**, 401 (1920).
12. Henderson, L. J., *The Blood*, New Haven, Conn., Yale University Press, 1928.
13. Henriques, O. M., *Biochem. Z.* **200**, 1,5,10,18,22, (1928); *Ergebn. Physiol.* **28**, 625 (1929); *J. Biol. Chem.* **29**, 1 (1931); *Biochem. Z.* **243**, 241 (1931); *Biochem. Z.* **260**, 58 (1933).
14. Margaria, R., and Green, A. A., *J. Biol. Chem.* **102**, 611 (1933).
15. Margaria, R., *J. Physiol.* **72**, 7 P, (1931); *J. Physiol.* **72**, 311 (1931); *Boll. Soc. It. Biol. Sper.* **7**, 425 (1932); *Schw. Med. Wochenschr.* **82**, 990 (1952).
16. Margaria, R., and Milla, E., *Boll. Sec. Biol. Sper.* **31**, 1250 (1955).
- 16A. Margaria, R., and Milla, E., Submitted for publication, 1956.
17. Meschia, G., and Barron, D. H., *Quart. J. Exp. Phys.* **41**, 180 (1956).
18. Milla, E., Giustina, G., and Margaria, R., *Giornale di Bioch.* **11**, 153; 434 (1953).
19. Milla, E., and Margaria, R., *Boll. Soc. It. Biol. Sper.* **30**, 475 (1954).
20. Roughton, F. J. W., *Physiol. Rev.* **15**, 241 (1935).
21. Roughton, F. J. W., *Harvey Lectures* **35**, 96 (1943/44).
22. Stadie, W. C., and Hawes, E. R., *J. Biol. Chem.* **77**, 265 (1928).
23. Van Slyke, D. D., Hastings, A. B., Heidelberger M., and Neill, J. M., *J. Biol. Chem.* **54**, 481 (1922).
- 23A. Van Slyke, D. D., and Hawkins, J., *J. Biol. Chem.* **87**, 265 (1930).
24. Van Slyke, D. D., Wu, and McLean, E. C., *J. Biol. Chem.* **56**, 765 (1923).
25. Van Slyke, D. D., Hastings, A. B., Murray, C., and Sendroy, J., Jr., *J. Biol. Chem.* **65**, 701 (1925).
26. Van Slyke, D. D., *J. Biol. Chem.* **52**, 525 (1922).
- 26A. Van Slyke, D. D., *Physiol. Rev.* **1**, 141 (1921).
27. Van Slyke, D. D., Hastings, A. B., and Neill, J. M., *J. Biol. Chem.* **54**, 507 (1922).
28. Wyman, J., *J. Biol. Chem.* **127**, 1; 581 (1939).



# The Role of Magnesium in the Body Fluids

J. Russell Elkinton

IN 1931, PETERS AND VAN SLYKE in their classic work *Quantitative Clinical Chemistry* (1) introduced their chapter on the subject of magnesium with a statement that up to that time no clinical significance had been attached to changes in magnesium metabolism. In 1944 in his unpublished revision of this chapter, Peters again stated that no pathologic conditions referable to magnesium metabolism had been discovered in man. In 1956, we are still not much wiser on the subject. Nevertheless knowledge on the broad subject of magnesium metabolism has been growing, and evidence is beginning to accumulate that magnesium may be an important factor in certain clinical situations. My excuse for talking about such a little-known subject can be stated as follows: In order to judge the necessity of a laboratory determination in a clinical situation and to interpret the results, the clinical chemist must be acquainted with the physiologic role of the chemical constituent involved and with the possible distortions of this physiologic role in disease. If this basic premise is accepted, the clinical chemist indeed has a wide realm of knowledge to make his own in order to become an expert in his field.

The ubiquitous distribution in nature and in the living organism of magnesium, like potassium, has always aroused the interest of biochemists and physiologists. Perhaps we can say that our knowledge of the role of magnesium in the body fluids is comparable to that of potassium 10 to 15 years ago. Certainly the explorations of the pathophysiology of magnesium have followed very closely those of potassium. It is therefore my plan to review briefly the problem of magnesium by making a few comparisons with its better known cationic brother, potassium.

## METHOD

The difficulty in measuring accurately magnesium in body fluids has always been a limiting factor in its investigation. In the older methods of precipi-

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tating magnesium as ammonium phosphate or hydroxyquinoline, the separation of calcium usually removed some of the magnesium (2, 3). More recently, the determination of magnesium by its combination with titan yellow has been widely used (4-7), and it is the method we are using at present in our own laboratory. Direct spectroscopy has been employed (8, 9). Newer methods also include determination by the use of versenes (10), or measurement in high-temperature flame photometers (11); as yet these methods have not been widely adopted. These advances in methodology, however, have made possible an acceleration of the study of magnesium metabolism, which is comparable in part to the same phenomenon in respect to potassium when the flame photometric method for determination of that cation was developed.

#### DISTRIBUTION IN THE BODY

Magnesium is a bulk metal in the living organism. Its position is predominantly in the bony skeleton and in the intracellular phases of soft tissues. This has been demonstrated by a variety of technics. Analyses of tissue such as skeletal muscle show that magnesium is the second highest cation in concentration in cell water (Table 1). The volume of distribution of in-

**Table 1.** AVERAGE CONCENTRATION OF PRINCIPAL CATIONS IN INTRACELLULAR WATER OF SKELETAL MUSCLE

Species	K	Mg (mEq./l. cell water)	Na	Investigators
Bat	161	34	9	Cotlove <i>et al.</i> <sup>28</sup>
Dog	161	26	10	Eichelberger and McLean <sup>68</sup>
Man	151	26	15	Mokotoff <i>et al.</i> <sup>69</sup>

jected magnesium has been studied by a variety of investigators, including Smith, Winkler, and Schwartz (12). These workers infused magnesium sulfate in dogs and found that, while its 4-hour distribution was roughly that of the extracellular fluid, namely, 20 to 25 per cent of the body weight, it was not recovered as rapidly as the sulfate over succeeding periods of time, and probably entered cells. In a few similar experiments in dogs, conducted in our department, with Clark and Barker (13), the volume of distribution of the magnesium varied widely and was considerably in excess of 20 per cent of the body weight (28 to 92 per cent) when calculated by dividing the amount of magnesium retained by the rise in concentration of diffusible, rather than of total, magnesium in plasma. These data again strongly suggest that part of the magnesium infused entered cells. The balance data also indicated that a smaller decrement of potassium occurred simultaneously. Recently the isotope,  $Mg^{28}$ , with a half-life of 21 hours, has been made available and has been used by Glicksman and co-workers of New York to study the distribution of mag-

nesium (14). Their findings are similar to those of the previous methods, namely, that magnesium is distributed in a portion of the body somewhat greater than the extracellular phase, but less than the calculated total body magnesium. It is quite clear, therefore, that magnesium like potassium is predominantly an intracellular constituent.

The amount of magnesium in the extracellular phase, and particularly in plasma, is at a much lower concentration than in cells, roughly one tenth of the intracellular concentration (Table 2). It is this portion that can be sampled so readily for clinical purposes. Like calcium, part of the magnesium is bound to plasma protein; the fraction so bound is roughly 25 to 35 per cent of the total plasma magnesium, and is smaller than the comparable fraction of calcium of approximately 50 per cent.

#### CELLULAR FUNCTION

Magnesium, like potassium, plays an essential role in cells as a metallic activator of enzyme systems (15, 16). It has been shown to activate transphosphorylases, phosphatases, enolase, keto acid carboxylase, and lecithinase. Thus it is a crucial factor in release of energy from ATP and in the carbohydrate metabolism of the organism.

#### PHARMACOLOGY

The pharmacologic effects of the magnesium ion have long been studied and recently have been reviewed in detail by Engbaek of Copenhagen (17). Magnesium is a nervous system depressant, both centrally and peripherally. Its effect on the central nervous system produces drowsiness and unconsciousness; in the peripheral nervous system it inhibits reflex activity and produces flaccid or curare-like paralysis; respiration is inhibited. Conversely, when magnesium is deficient or low, motor irritability is enhanced and symptoms much like tetany may be produced. The effect of magnesium on the cardiovascular system is likewise profound, although less critical than that on the nervous system. Cardiac conduction is depressed; the effect on the peripheral circulation is that of vasodilatation with depression of the blood pressure. These pharmacologic effects must be kept in mind when patients are considered who have received magnesium salts for therapeutic reasons.

#### ABSORPTION AND EXCRETION

Like potassium, magnesium is constantly available to the organism in ingested food, since magnesium is a metallic constituent of chlorophyll, occupying in this pigment the same central position that is held by iron in the heme pigments. Roughly, 20 to 40 mEq. of magnesium per day are ingested through the gastrointestinal tract, providing a daily turnover of approximately 1.5 to 3 per cent of total body magnesium. Of the magnesium ingested, two thirds of it is excreted in the feces. Balance studies by Tibbetts and Aub (18), McCance

Table 2. SERUM MAGNESIUM: DETERMINATIONS OF TOTAL CONCENTRATION AND NONDIFFUSIBLE FRACTION

Investigators	Total magnesium								Nondiffusible Mg. (%)			
	mg./100 ml.				mEq./l.*							
	n	Mean	-2σ	+2σ	Mean	-2σ	+2σ	n	Mean	Min.	Max.	
Phosphate precipitation methods												
Watchorn and McCance <sup>70</sup>	8	2.48	2.42†	2.59†	2.07	2.02†	2.16†	8	27	23	36	
Hald <sup>2</sup>	10	2.04	1.68†	2.88†	1.70	1.40†	2.40†					
Walker and Walker <sup>71</sup>	87	2.20	1.60†	3.00†	1.83	1.33†	2.50†					
Soffer <i>et al.</i> <sup>72</sup>	14	2.52	2.12†	4.30†	2.10	1.77†	3.58†	14	15	3	22	
Cope and Wolf <sup>60</sup>	17	1.99	1.75†	2.45†	1.66	1.46†	2.04†	17	37	14	56	
Bissell <sup>61</sup>	18	2.28	1.84	2.72	1.90	1.53	2.27	18	30	17	42	
Simonsen <i>et al.</i> <sup>73</sup>	42	2.01	1.69	2.33	1.68	1.41	1.94					
Stutzman and Amatuzio <sup>74</sup>	48	2.32	1.84	2.80	1.93	1.53	2.33					
Copeland and Sunderman <sup>75</sup>	17	2.12	1.68	2.50	1.77	1.40	2.08	17	35	23	48	
Hydroxyquinoline precipitation methods												
Greenberg <i>et al.</i> <sup>77</sup>	58	2.74	2.14	3.34	2.28	1.78	2.78					
Hoffman <sup>76</sup>	30	2.18	1.88	2.48	1.82	1.57	2.07					
Titan yellow methods												
Hirschfelder and Haury <sup>78</sup>	14	2.11	1.89	2.33	1.76	1.57	1.94	14	52			
Orange and Rhein <sup>6</sup>	45	2.27	1.90†	2.50†	1.89	1.58	2.08					
Flink <i>et al.</i> <sup>43</sup>	120	2.29	1.81	2.77	1.91	1.51	2.31					
Silverman and Gardner <sup>62</sup>	43	2.12	1.81	2.44	1.77	1.51	2.03	35	35	13†	56†	
Elkinton	41	2.13	1.69	2.57	1.78	1.41	2.16					

\*Equivalence calculated as if all Mg were ionized.

†Extreme values rather than + or - 2σ.

‡+ and - 2σ rather than extreme values. Plus and minus 2 standard deviations (σ) indicate the 95 per cent confidence limits of the distribution about the mean.

and Widdowson (19), and others indicate very definitely that fecal magnesium is primarily unabsorbed magnesium from food and is not secreted into the gut. When increments of magnesium are added to that ingested, most of it passes through and is excreted in the stool. Only a small portion is absorbed and excreted by the kidneys. This is the basis for the use of magnesium salts as cathartics. However, as mentioned below, there are exceptions to this general rule.

One third or less of the magnesium that is taken in each day is excreted by the kidney. Thus the amount of magnesium excreted by the kidney is very much smaller than that of potassium. Nevertheless, there is some evidence to suggest that renal mechanisms for handling magnesium may be similar to those

of potassium. Diffusible magnesium in plasma is filtered in the glomeruli and reabsorbed by the renal tubule. In addition, however, there is some rather equivocal evidence that magnesium may, like potassium, be *secreted* by the renal tubule. The high content of magnesium in the urine of the aglomerular goose fish is indubitable evidence that in that species magnesium is indeed excreted by a secretory process (20). In the mammalian glomerular kidney, however, it is a little more difficult to ascertain whether such a process exists. We have been studying the possibility of this phenomenon in our laboratories. In collaboration with Clark and Barker (13), it was shown that the constant infusion of magnesium in dogs in amounts that were just short of producing respiratory paralysis resulted in a high excretion rate of magnesium. When the amount of magnesium filtered was calculated from the ultrafiltrable concentration of magnesium in plasma, it was found that in some of the dogs a ratio of slightly above unity (1.1) was attained for magnesium excreted over magnesium filtered. The accomplishment of such a value above unity, however, depended upon the proportion of plasma magnesium which was freely diffusible, and ratios of 2 or more, such as those found with potassium, have not been reached. Therefore, although these data are suggestive, they do not provide any convincing evidence that magnesium is secreted by the renal tubule.

Very little is known of hormonal relationships to magnesium metabolism that are of clinical significance. A negative balance of magnesium has been reported in some, but not all, cases of hyperparathyroidism, with retention of the ion after parathyroidectomy (21, 22). In experimental animals parathormone has been found to increase the excretion of magnesium (23). In respect to the thyroid, although Tibbetts and Aub (24) found no abnormalities of the magnesium balance in patients with thyroid disease, the recent work of Tapley (25) indicates that a negative magnesium balance can be produced in myxedematous subjects by the administration of *l*-triiodothyronine. Pituitary-adrenal effects are not well known although a decrease in magnesium excretion following the administration of ACTH has been reported (26). It is clear that much remains to be learned concerning the effect of internal secretions on the metabolism of this ion.

#### DEFICITS AND EXCESSES OF MAGNESIUM

*Variations in magnesium distribution* in relation to that of other electrolytes have been studied experimentally. As Baldwin and co-workers (27) have shown by analysis of human tissues, conditions of primary deficits or excesses of potassium are usually accompanied by changes in magnesium content of muscle in the same direction. On the other hand, very little change occurred in the magnesium content of muscle of rats subjected to acid-base disturbances or to magnesium depletion by Cotlove and associates (28). Experimental loading of human subjects with sodium, and variation in the sodium output by manipulation of adrenocortical steroids by Hills, Parsons, Rosenthal, and Webster

(29) in our hospital, have produced data which indicate that magnesium is more likely to move with sodium than with potassium. This finding is in agreement with the work of Haynes, Crawford, and DeBailey (26), who made similar observations in respect to magnesium and sodium in the postoperative state. In our department we have found that potassium deprivation in normal human subjects may result in very little variation in the excretion of magnesium, despite wide variations in excretion of potassium (30). Experimental deprivation of magnesium in the normal human subject has recently been produced by Fitzgerald and Fourman (31) at Cardiff. Their data indicate that a small and transient increase in excretion of potassium accompanies the primarily induced magnesium deficit. Although massive magnesium loading in dogs, as observed in our own experiments mentioned above, would appear to produce some replacement of intracellular potassium by magnesium, magnesium and potassium do not seem to substitute for each other readily in cell fluid.

As in the case of potassium, much has been learned of the effects of *magnesium deficiency* by its production in experimental animals. The early experiments of Kruse, Orent-Keiles, and McCollum (32) on magnesium deficiency in rats indicated the profound effects that may be produced, namely, extreme vasomotor instability and neuromuscular irritability. In a number of animal species various pathologic lesions have been demonstrated, including hemorrhagic diatheses, myocardial fibrosis, and degenerative changes in kidney and central nervous system (33-36). Conservation of magnesium under conditions of deprivation has also been studied. The most complete study is that of Fitzgerald and Fourman referred to above, who found that during periods of approximately 3 weeks of deprivation, less than 2 mEq. per day of magnesium was excreted in the urine after some 8 days of the experiment; and about the same amount was found in the feces. It is apparent, therefore, that a renal conservation of magnesium can and does occur that is qualitatively if not quantitatively similar to that of potassium. It should be pointed out, however, that in certain pathologic conditions magnesium, again like potassium, is found in larger amounts in the urine at a time when the serum levels of the ion are low. Such data, found in respect to potassium by Tarail and myself (37) some 9 years ago, were interpreted to indicate that potassium was not conserved by the kidney. Subsequent investigations have amply shown that this is not so, but that the excretion of potassium in the urine under such circumstances is affected by either renal disease or hormonal influences, or both. The same is probably true of magnesium.

Clinical states involving magnesium deficiency have been described, although no clinical state has been proved to be due to magnesium deficiency alone. Again, as in the case of potassium, diabetic acidosis was early shown to involve a deficiency of magnesium. This was suggested by the work of Atchley and co-workers (38) at Columbia in 1933, and by the work of Butler *et al.* (39) in

Boston. More recently the complete balance study conducted by Nabarro, Spencer, and Stowers (40) at the University College Hospital in London, has provided a detailed knowledge of the relationship of these two intracellular cations in this condition. They found that both these cations as well as another intracellular constituent, phosphorus, were retained during treatment of diabetic acidosis, clearly indicating that cellular deficits had been present when the patient was admitted to the hospital. It is likewise of interest that the plasma levels of phosphorus and magnesium, as well as that of potassium, were elevated initially at a time when cellular stores were obviously depleted. As in the case of potassium, therefore, plasma levels of magnesium may not reflect the true state of the intracellular stores of the ion. Magnesium administration has not been shown to be as critical to the recovery of the patient from diabetic acidosis as has potassium. Nevertheless, it has led to the suggestion that magnesium should be incorporated in parenteral fluids given in this condition (39).

A low or absent intake of magnesium has been implicated as the cause of a deficiency or of low serum levels of magnesium in a variety of clinical states (41, 42). Flink and co-workers (43), and more recently Suter and Klingman (44), have reported the combination of a low serum magnesium level and neuromuscular hyperirritability and tremors, both in patients who have been on parenteral fluids containing no magnesium, and in patients with chronic alcoholism whose intake of normal foods has been greatly diminished. Both these types of patients seemed to be improved when magnesium was administered to them parenterally. Parenthetically it is of interest that hypomagnesemic tetany in cattle has recently been shown by Head and Rook (45) in Great Britain to be due to impaired intestinal absorption secondary to a high ammonia level in the rumen. Hypomagnesemia and neuromuscular irritability have been reported by Hirschfelder and others in patients with a variety of diseases, including glomerular nephritis, epilepsy, and hyperparathyroidism (46). That tetany can be caused by low levels of magnesium has long been suspected, but seldom proved in human subjects, since most cases also have low levels of calcium (47, 48). Furthermore, where hyperirritability has been improved by the administration of magnesium, there has been a much longer lag period than occurs when hypocalcemic tetany is treated with calcium. Magnesium deficiency due to excessive excretion rather than inadequate intake has been suggested to occur in patients with congestive heart failure who have received mercurial diuretics or cation exchange resins (49). As mentioned above, mercury will indeed increase the excretion of magnesium. Nevertheless, no critical role has been demonstrated to be played by magnesium deficiency in either congestive heart failure or in abnormal states resulting from its treatment.

It is thus apparent that magnesium deficiency has not been shown to be the direct cause of any specific pathologic condition in man. With the accumulation of knowledge of the biochemical role of magnesium, particularly as an



activator of important intracellular enzymes, it is highly probable that this state of affairs will not continue. There are many straws in the wind pointing to such an advance, such as the report of Gray and Jordan (50), who recently demonstrated that the administration of magnesium decreased the mortality of tourniquet shock in rats, apparently by inhibition of the decomposition of ATP. Magnesium may yet prove to be an important therapeutic agent, just as has potassium over the past decade.

*Excesses of magnesium* in the body have been studied in experimental animals. As indicated above, the pharmacologic effects of depression of the nervous and cardiovascular systems are well recognized. For this reason excesses of magnesium have eagerly been sought in clinical states. Toxic effects have been observed in patients who have been overtreated with magnesium, either parenterally or orally. Parenteral magnesium frequently has been given to combat hyperirritability and convulsions found in a variety of conditions, such as glomerulonephritis and eclampsia (51, 52). Occasionally patients with gastrointestinal lesions such as megacolon have been known to absorb undue amounts of magnesium given as a cathartic, with resultant nervous system depression (53). However, the commonest group of disease states in which hypermagnesemia is found is that involving renal insufficiency. High levels have been found in both acute and chronic renal disease, especially when associated with oliguria (46, 49, 54). When we know more of the precise renal mechanisms that are involved in the excretion of magnesium, we will be better able to understand the abnormalities observed in respect to this ion in various types of renal disease. Again, there is a striking parallel between the story of potassium and magnesium. Data obtained from some of our patients with acute renal failure indicate that, as in the case of potassium, the serum level of magnesium may be elevated during the oliguric phase and come down when the patient enters the diuretic phase and excretes an increasing amount of magnesium in the urine (55). The role of magnesium retention in the symptoms of uremia is a fascinating one to speculate upon.

*Abnormalities of internal distribution* of magnesium should be considered as well as absolute deficits and excesses of the ion. In the early days of the intense study of sodium and then of potassium, low or high levels of these ions in plasma were equated with deficits or excesses in the total body. We have long since learned that such relationships do not necessarily obtain. It is probable, therefore, that the same situation is true in respect to magnesium, and we should be alert to this possibility. As already indicated, a serum magnesium level may be high early in diabetic acidosis at a time when the magnesium stores of the body are low. In thyroid disease, evidence was put forward by Soffer *et al.* (58) and Dine and Lavieties (59) that there may be an abnormal distribution between the bound and unbound fractions of magnesium in plasma; subsequent observers have failed to confirm such an abnormality (60-62). Of more recent interest has been the exploration of the relationship of



**Table 3.** SUMMARY OF DIAGNOSTIC EVIDENCE INDICATING DISTURBED MAGNESIUM BALANCE

<i>Primary Mg disturbance</i>	<i>History</i>	<i>Clinical signs and symptoms</i>	<i>Biochemical signs</i>
Deficit	Low intake (Mg-free fluids postoperative); acute or chronic renal disease with polyuria; Hg diuresis; resin therapy; diabetic acidosis	Neuromuscular hyperirritability; tetany (?); anorexia (?)	Hypomagnesemia
Excess	High intake; renal insufficiency with oliguria	Neuromuscular, central nervous system, and cardiac depression	Hypermagnesemia

Modified from Elkinton and Danowski.<sup>79</sup>

magnesium distribution to changes in body temperature (63). It has been known for a long time that an elevation of plasma magnesium is characteristic of certain animals during hibernation (64, 65). With the recent development of hypothermia as an anesthetic and metabolic preparation for cardiac surgery, it may be possible that internal shifts of magnesium occur. Certainly in the experimental cooling of many animals, serum magnesium levels have been observed to rise (66, 67). This again is essentially *terra incognita* which awaits exploration by the physician, the physiologist, and the clinical chemist.

#### DIAGNOSIS OF MAGNESIUM IMBALANCE

The diagnostic evidence in this field on which the clinical chemist and the physician must operate is summarized in Table 3. Deficit of magnesium may occur primarily in situations where there is a low intake or an abnormal output of the ion. It should be looked for wherever there is an unusual state of neuromuscular hyperirritability or an unexplained tetany. At present the only readily available biochemical sign is the finding of hypomagnesemia. Conversely, excesses of magnesium may be present in patients who have an abnormally high intake of the ion for some therapeutic reasons, or in patients who have renal insufficiency, particularly with oliguria. It should be considered in any unusual state of neuromuscular, central nervous system, and cardiac depression. Again, the only biochemical sign available at present is that of a high level of magnesium in the plasma. Perhaps there should be added a third category of abnormal internal distribution of magnesium, but our ignorance of this kind of abnormality is so great that it can hardly be tabulated.

#### CONCLUSION

Our knowledge of the role of magnesium in the body fluids is steadily growing. Abnormalities of magnesium in disease conditions, however, are still

very little understood and are perhaps a decade or more behind our knowledge of the comparable roles of the other major ionic constituents. It would appear, therefore, that opportunity for advance in knowledge of the role of magnesium in disease states is great, and that the clinical chemist should be in the forefront of this advance.

## REFERENCES

1. Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry: I. Interpretations*. Baltimore, Williams & Wilkins, (1931), chap. 17
2. Hald, P. M., The determination of the bases of serum and whole blood, *J. Biol. Chem.* **103**, 471 (1933).
3. Greenberg, D. M., and Mackey, M. A., The determination of magnesium in blood with 8-hydroxyquinoline, *J. Biol. Chem.* **96**, 419 (1932).
4. Hirschfelder, A. D., and Serles, E. R., A simple adaptation of Kolthoff's colorimetric method for the determination of magnesium in biological fluids, *J. Biol. Chem.* **104**, 635 (1934).
5. Haury, V. G., Modification of titan yellow method for determination of small amounts of magnesium in biological fluids, *J. Lab. & Clin. Med.* **23**, 1079 (1938).
6. Orange, M., and Rhein, H. C., Microestimation of magnesium in body fluids, *J. Biol. Chem.* **189**, 379 (1951).
7. Heagy, F. C., The use of polyvinyl alcohol in the colorimetric determination of magnesium in plasma or serum by means of titan yellow, *Canad. J. Res.* **26**, sec. E, (1948).
8. Cassen, B., Method for rapid determination of magnesium in body fluids and some preliminary results on clinical material, *J. Lab. & Clin. Med.* **25**, 411 (1940).
9. Boyle, A. J., Whitehead, T., Bird, E. J., Batchelor, T. M., Iseri, L. T., Jacobson, S. D., and Myers, G. R., The use of the emission spectrograph for the quantitative determination of Na, K, Ca, Mg, and Fe in plasma and urine, *J. Lab. & Clin. Med.* **34**, 625 (1949).
10. Friedman, H. S., and Rubin, M. A., Clinical significance of the magnesium: calcium ratio: Technic for the determination of magnesium and calcium in biologic fluids, *Clin. Chem.* **1**, 125 (1955).
11. Davis, S., A flame photometric method for the determination of plasma magnesium after hydroxyquinoline precipitation, *J. Biol. Chem.* **216**, 643 (1955).
12. Smith, P. K., Winkler, A. W., and Schwartz, B. M., The distribution of magnesium following the parenteral administration of magnesium sulfate, *J. Biol. Chem.* **129**, 51 (1939).
13. Barker, E. S., Clark, J. K., and Elkinton, J. R., Unpublished studies.
14. Glicksman, A. S., Schwartz, M. K., Bane, H., Roberts, K. E., and Randall, H. T., Physiologic distribution and excretion of  $Mg^{28}$  in dogs and man, *Clin. Res. Proc.* **4**, 14 (1956).
15. Lehninger, A. L., Role of metal ions in enzyme systems, *Physiol. Rev.* **30**, 393 (1950).
16. Fruton, J. S., and Simmonds, S., *General Biochemistry*, New York, Wiley (1953), p. 803.
17. Engbaek, L., The pharmacological actions of magnesium ions with particular reference to the neuromuscular and the cardiovascular system, *Pharmacol. Rev.* **4**, 396 (1952).
18. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease: I. The magnesium and calcium excretion of normal individuals, also the effects of magnesium, chloride, and phosphate ions, *J. Clin. Invest.* **16**, 491 (1937).
19. McCance, R. A., and Widdowson, E. M., The fate of calcium and magnesium after intravenous administration to normal persons, *Biochem. J.* **33**, 523 (1939).

20. Forster, R. P., and Berglund, F., Osmotic diuresis and its effect on total electrolyte distribution in plasma and urine of the aglomerular teleost, *Lophius americanus*, *J. Gen. Physiol.* **39**, 349 (1956).
21. Bulger, H. A., and Gausmann, F., Magnesium metabolism in hyperparathyroidism, *J. Clin. Invest.* **12**, 1135 (1933).
22. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease: II. The effect of the parathyroid hormone, *J. Clin. Invest.* **16**, 503 (1937).
23. Roberts, B., Murphy, J. J., Miller, L., and Rosenthal, O., The effect of parathyroid hormone upon serum levels and urinary excretion of magnesium, *Surg. Forum, Am. Coll. Surg.* 509 (1954).
24. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease: III. In exophthalmic goiter, basophilic adenoma, Addison's disease and steatorrhea, *J. Clin. Invest.* **16**, 511 (1937).
25. Tapley, D. F., Magnesium balance in myxedematous patients treated with triiodothyronine: Preliminary note, *Bull. Johns Hopkins Hosp.* **96**, 274 (1955).
26. Haynes, B. W., Crawford, E. S., and DeBaakey, M. E., Magnesium metabolism in surgical patients: Exploratory observations, *Ann. Surg.* **136**, 659 (1952).
27. Baldwin, D., Robinson, P. K., Zierler, K. L., and Lilienthal, J. L., Jr., Interrelations of magnesium, potassium, phosphorus, and creatine in skeletal muscle of man, *J. Clin. Invest.* **31**, 850 (1952).
28. Cotlove, E., Holliday, M. A., Schwartz, R., and Wallace, W. W., Effects of electrolyte depletion and acid-base disturbance on muscle cations, *Am. J. Physiol.* **167**, 665 (1951).
29. Hills, A. G., Parsons, D. W., Rosenthal, O., and Webster, G. D., Jr., Observations of magnesium metabolism in man, *J. Clin. Invest.* **34**, 940 (1955).
30. Squires, E. D., Huth, E. J., and Elkinton, J. R., Unpublished data.
31. FitzGerald, M. G., and Fourman, P., An experimental study of magnesium deficiency in man, *Clin. Sc.* **15**, 635 (1956).
32. Kruse, H. D., Orent-Keiles, E. R., and McCollum, E. V., Studies on magnesium deficiency in animals: I. Symptomatology resulting from magnesium deprivation, *J. Biol. Chem.* **96**, 519 (1932).
33. Schrader, G. A., Prickett, C. O., and Salmon, W. D., Symptomatology and pathology of potassium and magnesium deficiencies in rat, *J. Nutrition* **14**, 85 (1937).
34. Moore, L. A., Hallman, E. T., and Sholl, L. B., Cardiovascular and other lesions in calves fed diets low in magnesium, *Arch. Path.* **26**, 820 (1938).
35. Greenberg, D. M., Anderson, C. E., and Tufts, E. V., Pathological changes in the tissues of rats reared on diets low in magnesium, *J. Biol. Chem.* **114**, 43 (Proc.) (1936).
36. Barron, G. P., Brown, S. O., and Pearson, P. B., Histological manifestations of a magnesium deficiency in the rat and rabbit, *Proc. Soc. Exp. Biol. & Med.* **70**, 220 (1949).
37. Tarail, R., and Elkinton, J. R., Potassium deficiency and the role of the kidney in its production, *J. Clin. Invest.* **28**, 99 (1949).
38. Atchley, D. W., Loeb, R. F., Richards, D. W., Jr., Benedict, E. M., and Driscoll, M. E., On diabetic acidosis; detailed study of electrolyte balances following the withdrawal and reestablishment of insulin therapy, *J. Clin. Invest.* **12**, 297 (1933).
39. Butler, A. M., Talbot, N. B., Burnett, C. H., Stanbury, J. B., and MacLachlan, E. A., Metabolic studies in diabetic coma, *Tr. A. Am. Physicians* **60**, 102 (1947).
40. Nabarro, J. D. N., Spencer, A. G., and Stowers, J. M., Metabolic studies in severe diabetic ketosis, *Quart. J. Med.* **21**, 225 (1952).
41. Martin, H. E., Edmondson, H., Homann, R., and Berne, C. J., Electrolyte problems

- in the surgical patient, with particular reference to serum calcium, magnesium and potassium levels, *Am. J. Med.* **8**, 529 (1950).
42. Levey, S., Abbott, W. E., Krieger, H., and Davis, J. H., Metabolic alterations in surgical patients: VIII. Studies involving iron and magnesium metabolism in patients with gastrointestinal drainage, *J. Lab. & Clin. Med.* **47**, 437 (1956).
  43. Flink, E. B., Stutzman, F. L., Anderson, A. R., Konig, T., and Fraser, R., Magnesium deficiency after prolonged parenteral fluid administration and after chronic alcoholism complicated by delirium tremens, *J. Lab. & Clin. Med.* **43**, 169 (1954).
  44. Suter, C., and Klingman, W. O., Neurologic manifestations of magnesium depletion states, *Neurology* **5**, 691 (1955).
  45. Head, M. J., and Rook, J. A. F., Hypomagnesaemia in dairy cattle and its possible relationship to ruminal ammonia production, *Nature* **176**, 262 (1955).
  46. Hirschfelder, A. D., Clinical manifestations of high and low plasma magnesium; dangers of epsom salt purgation in nephritis, *J.A.M.A.* **102**, 1138 (1934).
  47. Hirschfelder, A. D., Clinical manifestations of hypo- and hyper-magnesaemia, *J. Clin. Invest.* **12**, 982 (1933).
  48. Miller, J. F., Tetany due to deficiency in magnesium; its occurrence in a child of 6 years with associated osteochondrosis of capital epiphysis of femur (Legg-Perthes disease), *Am. J. Dis. Child.* **67**, 117 (1944).
  49. Martin, H. E., Mehl, J., and Wertman, M., Symposium on recent advances in medicine; Clinical studies of magnesium metabolism, *M. Clin. North America* **36**, 1157 (1952).
  50. Gray, I., and Jordan, W. J., Magnesium protection in mechanical trauma, *Arch. Biochem. & Biophysics* **57**, 521 (1955).
  51. Winkler, A. W., Smith, P. K., and Hoff, H. E., Intravenous magnesium sulfate in the treatment of nephritic convulsions in adults, *J. Clin. Invest.* **21**, 207 (1942).
  52. Pritchard, J. A., The use of the magnesium ion in the management of eclamptogenic toxemias, *Surg. Gyn. & Obst.* **100**, 131 (1955).
  53. Collins, E. N., and Russell, P. W., Fatal magnesium poisoning following magnesium sulfate, glycerin and water enema in primary megacolon, *Cleveland Clin. Quart.* **16**, 162 (1949).
  54. Haury, V. G., and Cantarow, A., Variations of serum magnesium in 52 normal and 440 pathologic patients, *J. Lab. & Clin. Med.* **27**, 616 (1942).
  55. Bluemle, L. W., Jr., Potter, H. P., and Elkinton, J. R., Unpublished data.
  56. Hamburger, J., *Clin. Chem.* **3**, Supp., 332 (1957).
  57. Hamburger, J., and Richet, G., Enseignements tirés de la pratique du rein artificiel pour l'interprétation des désordres électrolytiques de l'urémie aiguë, *Rev. française d'études clin. et biol.* **1**, 39 (1956).
  58. Soffer, L. J., Cohn, C., Grossman, E. B., Jacobs, M. D., and Sobotka, H., Magnesium partition studies in Graves' disease and in clinical and experimental hypothyroidism, *J. Clin. Invest.* **20**, 429 (1941).
  59. Dine, R. F., and Laviertes, P. H., Serum magnesium in thyroid disease, *J. Clin. Invest.* **21**, 781 (1942).
  60. Cope, C. L., and Wolff, B., The ultrafilterable serum magnesium in hyperthyroidism, *Biochem. J.* **36**, 413 (1942).
  61. Bissell, G. W., The magnesium partition in hyperthyroidism with special reference to the effect of thiouracil, *Am. J. Med. Sc.* **210**, 195 (1945).
  62. Silverman, S. H., and Gardner, L. I., Ultrafiltration studies on serum magnesium, *N. England J. M.* **250**, 938 (1954).
  63. Heagy, F. C., and Burton, A. C., Effect of intravenous injection of magnesium chloride on the body temperature of the unanesthetized dog, with some observations on magnesium levels and body temperature in man, *Am. J. Physiol.* **152**, 407 (1948).

64. Suomalainen, P., Production of artificial hibernation, *Nature* **142**, 1157 (1938).
65. Riedesel, M. L., and Folk, G. E., Jr., Serum magnesium and hibernation, *Fed. Proc.* **15**, 151 (1956).
66. Platner, W. S., and Hosko, M. J., Jr., Mobility of serum magnesium in hypothermia, *Am. J. Physiol.* **174**, 273 (1953).
67. Axelrod, D. R., and Bass, D. E., Electrolytes and acid-base balance in hypothermia, *Am. J. Physiol.* **186**, 31 (1956).
68. Eichelberger, L., and McLean, F. C., The distribution of calcium and magnesium between the cells and the extracellular fluids of skeletal muscle and liver in dogs, *J. Biol. Chem.* **142**, 467 (1942).
69. Mokotoff, R., Ross, G., and Leiter, L., The electrolyte content of skeletal muscle in congestive heart failure; A comparison of results with inulin and chloride as reference standards for extracellular water, *J. Clin. Invest.* **31**, 291 (1952).
70. Watchorn, E., and McCance, R. A., Inorganic constituents of cerebrospinal fluid: II. The ultrafiltration of calcium and magnesium from human sera, *Biochem. J.* **26**, 54 (1932).
71. Walker, B. S., and Walker, E. W., Normal magnesium metabolism and its significant disturbances, *J. Lab. & Clin. Med.* **21**, 713 (1936).
72. Soffer, L. J., Dantes, D. A., Grossman, E. B., Sobotka, H., and Jacobs, M. D., Ultrafiltrable magnesium in hyperthyroidism, *J. Clin. Invest.* **18**, 597 (1939).
73. Simonsen, D. G., Westover, L. M., and Wertman, M., The determination of serum magnesium by the molybdivanadate method for phosphate, *J. Biol. Chem.* **169**, 39 (1947).
74. Stutzman, F. L., and Amatuzio, D. S., A study of serum and spinal fluid calcium and magnesium in normal humans, *Arch. Biochem.* **39**, 271 (1952).
75. Copeland, B. E., and Sunderman, F. W., Studies in serum electrolytes: XVIII. The magnesium-binding property of the serum proteins, *J. Biol. Chem.* **197**, 331 (1952).
76. Hoffman, W. S., A colorimetric method for the determination of serum magnesium based on the hydroxyquinoline precipitation, *J. Biol. Chem.* **118**, 37 (1937).
77. Greenberg, D. M., Lucia, C. P., Mackey, M. A., and Tufts, E. V., The magnesium content of the plasma and the red corpuscles in human blood, *J. Biol. Chem.* **100**, 139 (1933).
78. Hirschfelder, A. D., and Haury, V. G., Variations in magnesium and potassium associated with essential epilepsy, *Arch. Neurol. & Psychiat.* **40**, 66 (1938).
79. Elkinton, J. R., and Danowski, T. S., *The Body Fluids: Basic Physiology and Practical Therapeutics*, Baltimore, Williams & Wilkins (1955), p. 486.

# Electrolyte Disturbances in Acute Uremia

*Jean Hamburger*

IF THE CONSERVATIVE TREATMENT OF ANURIA has been so effective during the past few years, it is largely due to a better understanding of the electrolyte disturbances in acute uremia and to a clearer insight into the methods of control and correction of these disorders. There remains, however, much to be done in this field, for much is still obscure. And it is a fruitful field, for every new discovery has not only an academic interest but has also practical consequences enabling us to do still more for our patients.

## METHODS

Our contribution to this problem is based on both clinical and experimental findings.

### CLINICAL

The clinical findings concern 220 cases of anuria from different causes. In all these cases, one or several "ionograms" were taken for the levels of sodium, potassium, calcium, magnesium, chloride, bicarbonate, phosphate, sulfate, and protein. The organic acid levels were calculated by subtraction, except in some cases which I will mention later (Fig. 1). In the course of 150 sessions of dialysis with the artificial kidney, particular importance was paid to the comparison between the fluctuations in levels of these various electrolytes (at the beginning and end of the session) and the accompanying changes in the clinical state, thus endeavoring to correlate the particular clinical response with each electrolyte upset.

### EXPERIMENTAL

The experimental findings concern 200 rats and 39 dogs rendered anuric by ligation of the ureters. With the rat we developed a method of ligating the ureters in two stages with the object of avoiding interference with the effect of the anuria by operative shock (Fig. 2). Young rats were taken and their growth curves were plotted for at least 3 weeks. The first stage consisted in

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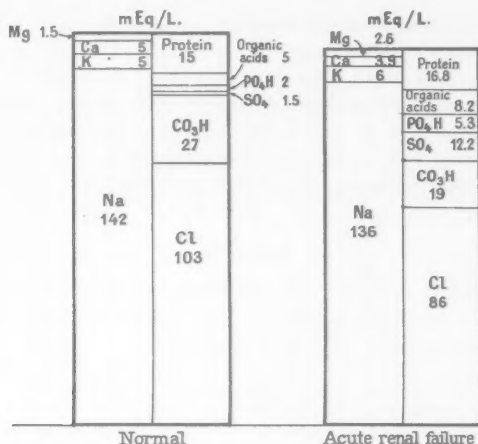


Fig. 1. Electrolyte structure of plasma in acute renal failure. Average electrolyte concentration in 60 cases of anuria with more than 400 mg. of blood urea/100 cc.

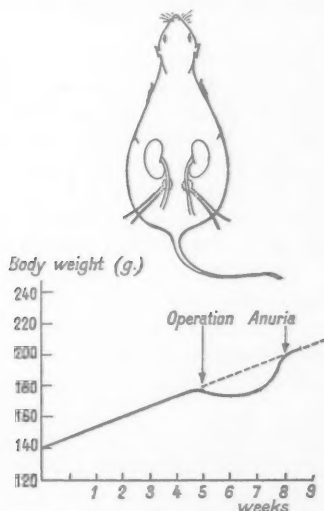


Fig. 2. Method of ligating the ureters of young rats in two stages to avoid operative shock. *First stage:* Passing a thread loosely around each ureter with the two ends coming out through the lumbar musculature. *Second stage:* To ascertain the disappearance of operative shock, one waits until the growth curve has regained the preoperative slope. When this occurs, the threads are tied closing off the ureters.

passing a thread loosely round each ureter with the two ends coming out through the lumbar musculature of the animal's back. The animals were allowed to recover completely from this operation. To estimate when all trace of operative shock had disappeared we waited until the growth curve had regained the slope that it had before operation. When this occurred the threads were coiled and tightened, closing off the ureters without a further operation, thus causing anuria without the phenomena of shock being superimposed.

## RESULTS

We will examine in turn the results observed for (1) sodium, (2) potassium, (3) calcium, (4) magnesium, and (5) the anions, namely, chloride, bicarbonate, phosphate, sulfate, and the organic acids.

### SODIUM

It is classic to describe a slight lowering of the plasma sodium level in anuria, even when there is no apparent loss, no vomiting or diarrhea. Our findings confirm this. The average natremia in 150 anuric subjects having no alimentary losses was 136 mEq./l., against a normal level of 142 mEq./l. Thus there was a slight lowering; this does not usually occur in patients whom we are observing at present.

The reason for this lowering of the plasma sodium has long intrigued doctors. Some have proposed intracellular shifts of sodium. Our observations suggest a direct relationship between the degree of hydration of the body and the level of the plasma sodium. Experimentally, this relationship stands out strikingly in the comparison between the sodium levels in two groups of anuric rats, one group receiving no external fluids and the other group receiving fluids equivalent to 14 per cent of their weight. In the first group the plasma sodium is above the normal and in the second below (Fig. 3). With an anuric patient it is not always so easy to establish an exact record of water intake and output because the patient is only rarely hospitalized from the beginning of the anuria. Furthermore, in cases in which the fluid administered has been calculated to compensate for the extrarenal water losses, there is still a tendency to a lowered plasma sodium. But let us examine more closely what happens in such a case.

The patient has been given a volume of water which appears to be equal to the volume eliminated in the feces and by the so-called "insensible" losses; perfect water balance is believed to have been achieved since the weight of the patient remains stable, and yet the hydration of the body is in fact increased by a significant amount. This phenomenon can be demonstrated in two ways: (1) If one follows the weight curve one sees it fall sharply by about 10 kg. at the time of the diuresis (Fig. 4). This loss of weight takes place at the moment the patient improves and the nitrogen balance shows that the intense cata-



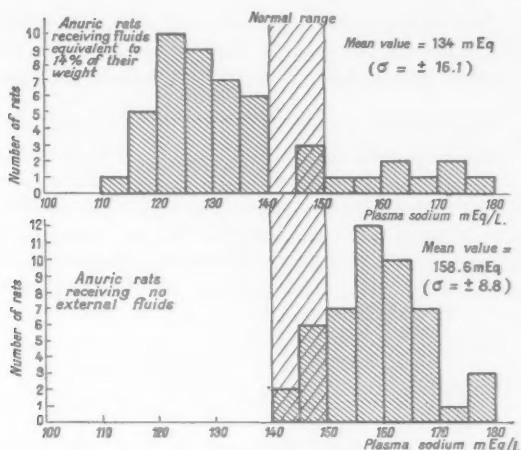


Fig. 3. Comparison between the plasma sodium in two groups of anuric rats, one group receiving no external fluids (48 rats) and the other group receiving fluids equivalent to 14 per cent of their weight (49 rats).

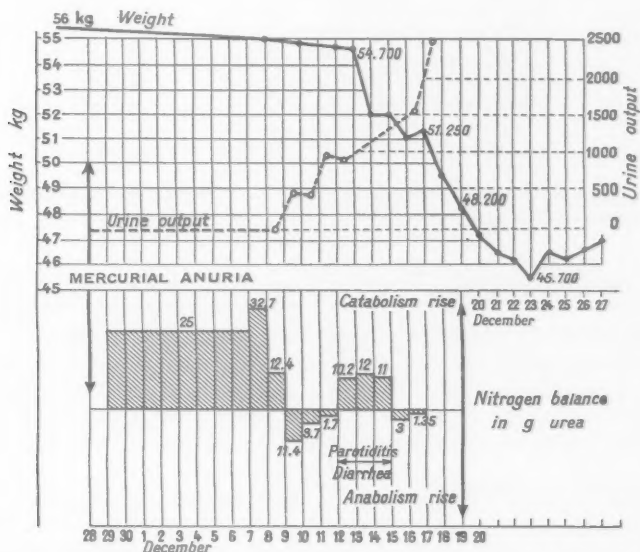


Fig. 4. Weight curve, daily urine output, and nitrogen balance in case of mercurial anuria.

bolism of the early days is giving way to an anabolism which will rebuild the wasted muscles. How can one explain this "delayed wasting" if not by the elimination of water accumulated in excess in the body during the period of anuria?

(2) In man and in dogs we have been able to obtain still more direct evidence of this increase in total hydration in spite of an apparently satisfactory balancing of the fluid intake. The measurement of the diffusion space of deuterium at the beginning of the anuria and again 4 days later has showed us an increase in this space in every case, this increase being on an average of 8 per cent (Fig. 5). It is very difficult to know exactly the distribution of this excess water between the intracellular and extracellular compartments, because

<i>Dog No. 31</i>	<i>Before ligature</i>	<i>3 rd day</i>
$D_2O$ space (ml.)-----	4.500	5.300
(per cent)-----	74	80
Muscle water (per cent)---	73	74
Thiocyanate space (ml.)---	991	866
(per cent)---	16	14.5
Effective osmotic pressure of plasma (milliosm. /L.)---	319	315
Mean corpuscular volume-----	70	73

Fig. 5. The diffusion space of deuterium increases in anuric dogs in spite of an apparently satisfactory balancing on the fluid intake.

the methods for measuring the extracellular space by inulin and similar substances, already of debatable value in the healthy subject, are certainly of no value in the anuric patient. One can only say that the absence of signs of hemodilution and the absence of obvious increase in the diffusion space of sodium thiocyanate suggest that at least a good part of this excess water would be found in the intracellular compartment. This water must be derived from the following sources (the calculations are illustrated in Fig. 6).

#### FROM PROTEIN CATABOLISM

The endogenous urea production averaged 240 Gm. The water of combustion would be 280 ml. and the pre-existing water liberated 2100 ml., making an average total of 2400 ml. of water from this source.

## FROM GLUCOSE CATABOLISM

Our patients were given 250 Gm. of glucose a day, an average of 1800 Gm. during the period of anuria; 1000 ml. of water would be derived from this.

## FROM FAT CATABOLISM

Deducting the weight loss accounted for by protein catabolism, it would appear that 6.8 kg. of fat were burned. The water of combustion would be 4900 ml. and the existing water liberated 2400 ml., giving a total from this source of 7300 ml.

From these three sources the patients derived on the average 10 liters of water. This figure agrees well with the weight loss at the onset of diuresis. Part is made up of water liberated from tissue breakdown (4000 ml.), but the

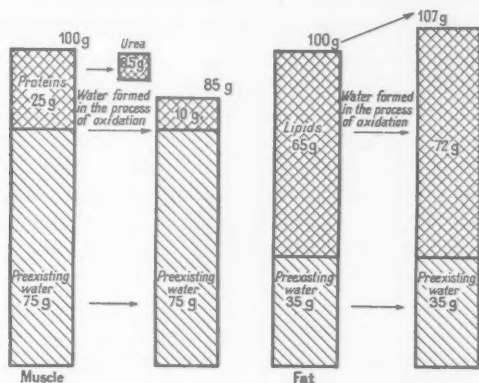


Fig. 6. Preexisting water and water of oxidation derived from 200 Gm of muscle and fat, respectively.

greater part (6000 ml.) is water formed in the body as a result of the increased catabolism during anuria.

This shows that this tendency to increased hydration in the anuric patient is certainly responsible for the tendency to a lowered plasma sodium level. Thus, since we have been further restricting the fluid intake so as to allow the patient to lose weight in proportion to his nitrogen catabolism, the plasma sodium levels of our patients usually remain strictly normal throughout their illness.

In brief, the plasma sodium appears to us to be lowered only in cases of increased hydration. Its level would appear to be a good method of control to reveal an overload of water, especially a risk of cellular overhydration.

The clinical symptoms which are associated with this hyponatremia are es-

entially the symptoms of overhydration, and although we have devoted the last 5 years of research to the examination of these symptoms of overhydration, it is beyond the bounds of the subject to speak about them.

#### POTASSIUM

Many workers have written that the level of plasma potassium is almost always raised in anuria. Hoff, Smith, and Winkler (1941) have stated that in a dog in acute renal insufficiency the rise in the plasma potassium was constant, progressive, and rapid. Our results contradict this statement. The average of all our results provides a figure which is slightly above the normal, 6 mEq./l. instead of 5. But if we examine the results in detail, only 22 per cent have a frankly raised potassium level at or above 7 mEq./l., 63 per cent have a normal level, and 15 per cent have a level even lower than 4 mEq./l., which is definitely low (Fig. 7). A raised potassium level is thus variable and not constant in

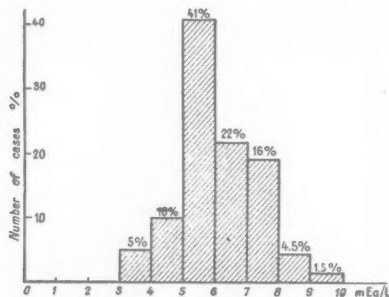


Fig. 7. Plasma potassium in anuria.

anuria. Similar results have been noted by Harris, McDonald, and Williams (1952). The contradiction is perhaps only apparent between these results and the classic notions; it is probably explained by the following facts: (1) at the present time the general condition of anuric patients is almost always good, which was not the case some years ago before the recent advance in conservative treatment, and states of severe malnutrition tend to encourage hyperpotassemia; (2) the glucose intake in the diet of our anuric patients is relatively large, which appears to facilitate the lowering of the potassium levels.

The clinical manifestations associated with the variations in the potassium levels, notably the electrocardiographic changes, appeared to be similar to those described by all other workers. However, there is no exact relationship between the degree of hyperpotassemia and the amount of the changes in the electrocardiographic tracing.

## CALCIUM

With about one exception, the level of plasma calcium was constantly low in our anurias. The general average was 3.9 mEq./l. of plasma. This finding is in accordance with the clinical observations of Bradley (1946) and others, and with the experimental findings of Mason *et al.* (1937), and also those of Harris, McDonald, and Williams (1952).

It is remarkable that this hypocalcemia was not once accompanied by signs of tetany. Perhaps the usual acidosis of these patients is the explanation of this fact.

## MAGNESIUM

The magnesium was estimated by a colorimetric method originally devised by Masson (1955) in my laboratory using titanium yellow. This method provided a normal value of 1.5 mEq./l. of plasma ( $\pm 0.25$ ).

In anuria, as much as a raised potassium has appeared to us to be inconstant, so a raised magnesium has been constantly found without exception (in more than 250 estimations). The average level was 2.6 mEq./l., that is, 174 per cent of normal. In 23 per cent of anuric patients the magnesium level was above 3 mEq./l. The highest was observed in a very grave anuria following the inhalation of carbon tetrachloride and reached 6.3 mEq./l. Contrary to certain previous statements, we found no relationship between this raised plasma magnesium and the level of potassium or calcium.

Two clinical signs have appeared to us to follow fairly closely the level of magnesium in our patients. These signs disappeared according to the reduction of this level by the artificial kidney. These signs were: (1) clouding of consciousness (thus, coma was complete in our patient with carbon tetrachloride poisoning with the extreme magnesium level of 6.3 mEq./l., and he came out of coma as soon as the magnesium was returned to normal levels by dialysis, without changes in the other electrolytes appearing capable of explaining clearly this return of consciousness); and (2) prolongation of the QT interval on the electrocardiogram (Table 1), which followed changes in the

**Table 1.** MODIFICATION OF THE Q-T INTERVAL DURING DIALYSIS WITH THE ARTIFICIAL KIDNEY

	S...		L...		T...	
	Before	After	Before	After	Before	After
Q-T interval* (sec.)	0.45	0.36	0.45	0.35	0.44	0.31
Heart rate (per min.)	136	158	75	75	88	100
Plasma potassium (mEq./l.)	3.5	4.1	5.1	4.2	7.7	6.4
Plasma calcium (mEq./l.)	4.8	7.1	3.9	6.2	3.5	5.5
Plasma magnesium (mEq./l.)	3	1.7	2.9	1.5	3.4	1.6

\*Corrected after Bazett (H. C.): An analysis of the time relation of electrocardiograms (*Am. Heart J.* 7, 353 (1918-1920, 7, 353)).

magnesium level where the levels of potassium and calcium appeared to have no effect.

### ANIONS

The disturbance of the anion equilibrium is one of the most constant and most characteristic biochemical change in acute renal insufficiency. It is shown by Fig. 8.

1. Lowered chlorides, averaging 86 mEq./l.
2. Bicarbonates usually lowered (average 19 mEq./l., the range being from 7.7 to 34.2 mEq./l.); the rare cases in which the bicarbonates were raised responded almost always to chloride losses by vomiting or gastric aspiration.
3. The sulfates were constantly and considerably raised. The average being 12.2 mEq./l., a figure eight times the normal. In 20 per cent of cases the sulfate level rose above 15 mEq./l., and we observed figures above 20 mEq., which in one instance reached 44 mEq. (without any administration of sulfate being responsible). It is remarkable that such a striking abnormality of the plasma of anuric patients has so rarely been recorded although it had been already reported in 1927 by Loeb and Benedict.
4. The phosphates were usually raised (the average being 5.3 mEq./l., that

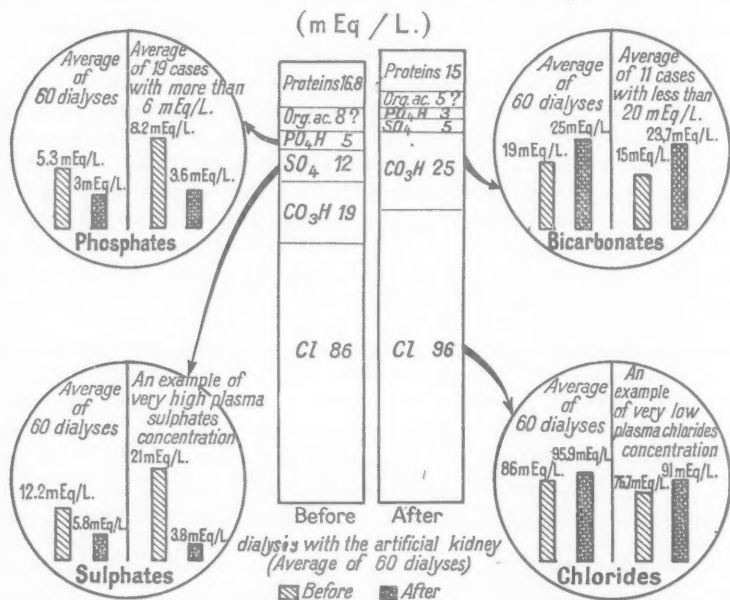


Fig. 8. Plasma anions in 60 anuric patients treated with the artificial kidney.

is, nearly three times normal), but to a variable degree and sometimes not at all. It is impossible to decide on the reasons for such variations; Harris (1952) believed that he could relate them to the sulfate level, which is in direct contradiction with our own findings.

5. The organic acids were slightly increased. In truth, this statement is made with certain reservations; it is based only on: (a) the questionable estimations of the total organic acids by the difference between the column of cations and that of all the other anions, and (b) the level of total organic acids by a process applying to plasma the method devised by Van Slyke for urine, a process of which I do not know the precise value. The realm of organic acids is certainly upset in acute renal insufficiency, as Nordmann has demonstrated and shown by many chromatographic determinations on the blood and urine of my patients. But it is a little premature to draw up a scheme of these changes.

But if one takes into account what one knows already, it is clear that the disturbance of the anions occupies an essential place in the biochemistry of acute renal insufficiency. This stands out particularly if one draws up a list of electrolyte levels in percentages of the normal (Fig. 9). One observes especially

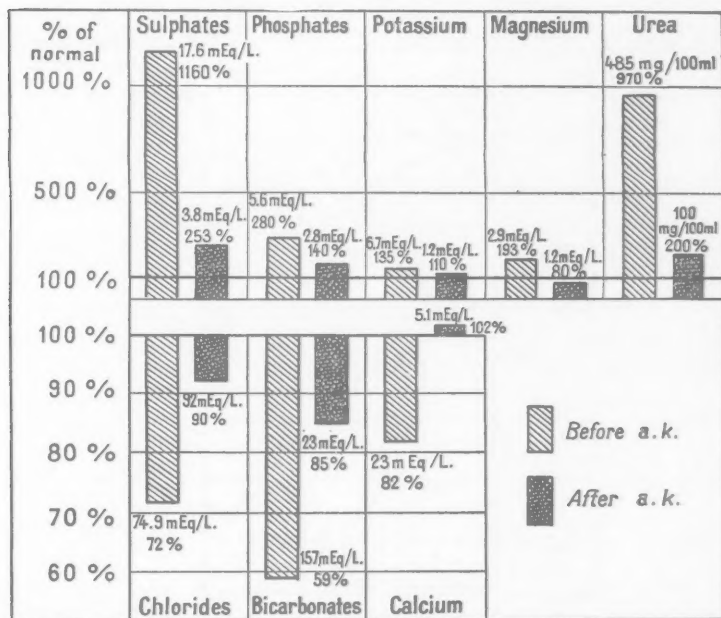


Fig. 9. Plasma electrolyte percentage of normal in a case of mercurial anuria treated with the artificial kidney.

that the percentage of sulfate retention is on the whole very exactly proportional to that of the retained nitrogen.

What is the clinical result of these anion disturbances? It is difficult to analyze. But a strong argument suggests that these may be of greater importance than had been thought up to the present, and this appears from the results obtained from the use of the artificial kidney. As have other workers, we have been struck by the surprising improvement in the general condition of patients suffering from acute uremia even during the session of dialysis. Since using this procedure, our anuric patients no longer have any of the functional, so-called "uremic," symptoms. They get up each day, are mentally clear, and have satisfactory appetites. The extent of this therapeutic response can hardly be compared with the partial and variable improvement that we obtained previously by peritoneal or intestinal dialysis. But if one compares the biochemical effect of the two types of procedure, one notes that their action differs only slightly on the retained nitrogen and the column of cations (excepting calcium), but that they act very differently on the anion balance. Intestinal perfusion, for example, did not correct the high sulfate and high phosphate

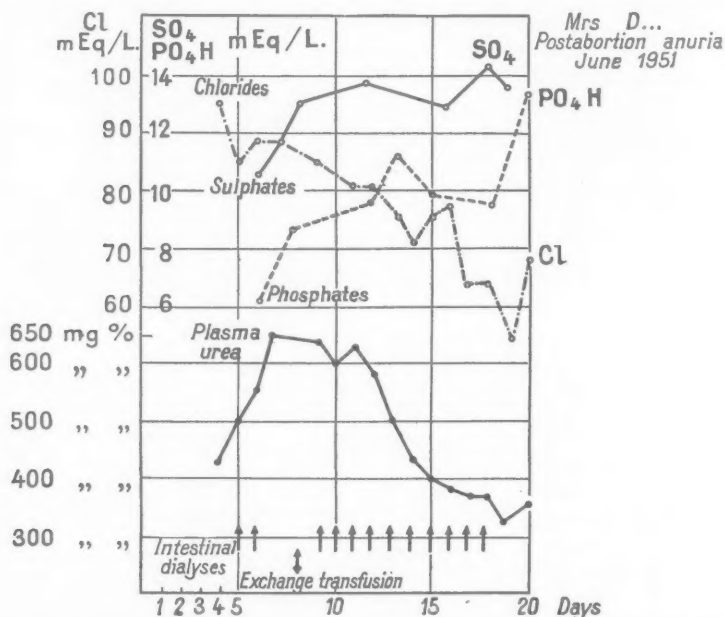


Fig. 10. Intestinal dialysis did not correct the high sulfate and high phosphate levels, whilst they are corrected by the artificial kidney.



levels (Fig. 10), while these were perfectly corrected, as has been seen in the preceding figures, by the artificial kidney. It appears, therefore, legitimate to relate the remarkable action of the artificial kidney on the anion disturbances with its spectacular effect on the general condition of the patients, and to ask whether one has not failed to recognize up to the present the responsibility of these anion disturbances (notably the high sulfate and high phosphate levels) for the clinical disorders in acute uremia.

### CONCLUSION

Nearly 150 years ago Laennec discovered auscultation and formed the basis of what he called "La méthode anatomo-clinique," that is to say the search for a systematic correlation of symptoms and lesions. Today, a new but similar task has arisen that one might call the biochemical-clinical method, which consists of the systematic correlation of biochemical disorders and their clinical manifestations.

### REFERENCES

1. Bradley, S. E., Biochemical abnormalities during renal insufficiency, *New England J. Med.*, **235**, 755, 791 (1946).
2. Hamburger, J., and Richet, G., Enseignements tirés de la pratique du rein artificiel pour l'interprétation des désordres électrolytiques de l'urémie aiguë (Rev. franç. *Études clin. et biol.* **1**, 39 (1956).
3. Harris, H., McDonald, I. R., and Williams, W., The electrolyte pattern in experimental anuria, *Australian J. Exper. Biol. Med. Sc.* **30**, 51 (1952).
4. Hoff, H. E., Smith, P. K., and Winkler, A. W., The cause of death in experimental anuria, *J. Clin. Invest.* **20**, 607 (1941).
5. Mason, M. F., Resnik, H., Minot, A. S., Rainey, J., Pilcher, C., and Harrison, T. R., Mechanism of experimental anuria, *Arch. Int. Med.* **60**, 312 (1937).
6. Masson, M., Crosnier, J., and Richet, G., Analyse de 93 ionogrammes dans l'insuffisance rénale. Contribution à l'étude du magnésium et des sulfates du plasma, *J. Urologie* **61**, 333 (1955).

# Blood Electrolytes Under the Influence of Cortical Hormones

R. Neher

**T**HE SIGNIFICANT INFLUENCE OF THE ADRENAL CORTEX on electrolyte metabolism in man was first widely recognized in 1932. Since then all aspects of the subject have, as a result of the works of Loeb (1, 2), and associates been intensively studied (3, 4). The development progressed from the use of adrenal cortical extracts (5) to the isolation and synthesis of adrenal cortical hormones (6-9) such as corticosterone, desoxycorticosterone (cortexone, DOC), cortisone, and hydrocortisone. A new impulse was given to it by the recent discovery of aldosterone (10, 10a) and new synthetic analogs of the natural hormones (11-14).

## METHOD

One can only arrive at a reasonable understanding of the interaction of blood electrolytes and adrenal cortical hormones by following the fate of the blood electrolytes in the places where they can diffuse freely out of the plasma, and in secretions which are important for diagnoses, like urine, saliva, and sweat. In other words, one should examine every site at which the adrenal cortical hormones produce on the electrolytes an easily observed activity that is more or less marked. In doing this, we have confined ourselves to studies in human subjects.

## ELECTROLYTE DISTRIBUTION

With this in view, we have first to clarify and determine how, and in what amounts, these electrolytes are distributed in the organism, together with similar data about the water accompanying them as solvent. Ever since it was recognized that Na and Cl occur chiefly in the blood plasma and interstitial

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fluid, as opposed to K,<sup>1</sup> in the cells, the total body water has been classified into two compartments in a gross oversimplification, these being the extracellular and intracellular fluid spaces (15). We want to see now more exactly how the electrolytes distribute themselves in the various body fluids. Figure 1 shows ionograms of blood serum, and interstitial and intracellular fluids (according to 16, 17, with modified presentation) where the volume is plotted as abscissa against the ion concentration in mEq./l., the resulting area representing the total electrolyte content. The 60 per cent of man's total body weight con-

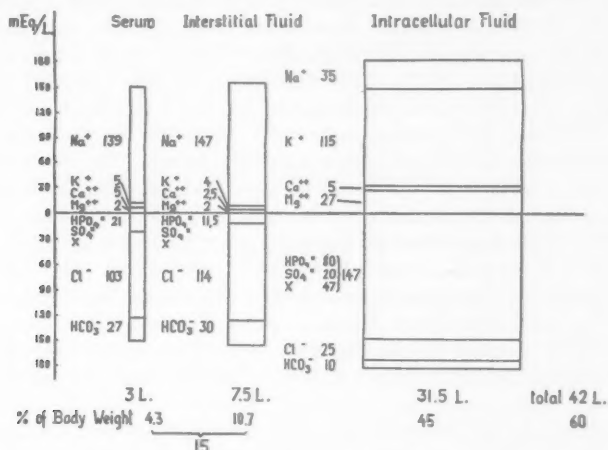


Fig. 1. Modified ionograms of blood serum, interstitial and intracellular fluids, and distribution of body water in man. *Abscissa*: volume in liters. *Ordinate*: ion concentration in mEq./l. (according to values from 16-20).

stituting water is three-fourths intracellular, and one-fourth extracellular (18-20).

Serum and interstitial fluid are very like one another in composition, but very different from the intracellular fluids, which contains, apart from a higher electrolyte concentration, the contrasting proportions of Na, K, and anions already mentioned. Because of these facts, a change of the Na concentration in the extracellular fluid can be determined representatively in the serum, but in the case of K, localized intracellularly to over 98 per cent, the excess or deficit cannot be derived simply from the serum content. More exact criteria can only be obtained here by the balance method, this being true to a lesser extent for Na too. In saliva (21), sweat (22), and urine (16) Na

<sup>1</sup>The designations Na, Cl, K, P, etc. refer throughout to the ions of sodium, chloride, potassium, phosphate, etc.

still predominates, as in the extracellular fluid, though not on anything like the same scale, and the total electrolyte concentration is, furthermore, lower than in the serum—this remark applying especially to the saliva and sweat. Large differences in the Na and K distribution are not only to be found between intracellular and extracellular space, but also between particular tissues, for instance, the Na:K ratio in skeletal muscle is about 0.3, in bone about 7.3, and in skin about 4.4 (23).

#### HOMEOSTASIS

It is remarkable that although the electrolyte content differs qualitatively and quantitatively in the various parts of the body, all these individual concentrations are day by day maintained constant in spite of the relatively large variation in electrolyte intake. The importance of this constancy for the proper functioning of all living cells had been noticed by Claude Bernard as early as 1878 (24). The kidneys are largely responsible for the maintenance of this homeostasis, together with some extrarenal factors. The electrolyte and water excretion is so regulated by the kidneys that the losses from the combined excretions of the organism remain normally in equilibrium with the intake. Table 1 illustrates what an enormous amount of work the kidneys have to do in order to accomplish this (16, 17). The values show a high filtration rate for Na and Cl, both present in high concentration in the serum, and the considerably lower filtration of K and P which possess a much lower serum level. In this connection it is worth remembering that it requires only small changes in the tubular reabsorption to cause relatively large changes in the Na excretion.

The capacity of the kidneys to carry out these vital functions is controlled by a number of endocrine factors, particularly important amongst which are

Table 1  
Renal Water and Electrolyte Excretion. (16,17)

	Average Excretion in Urine g./24 h.	Glomerular Filtrate g./24 h.	Tubular Reabsorption of Electrolytes % of Filtrate
Na	2.55	587.88	99.6
K *	2.34	35.1	93.4
Chloride	4.22	658.17	99.4
Phosphate (as P)	0.93	5.58	83.4
Sulfate (as S)	0.73	2.88	74.4
Water	1.2 L.	180 L.	99.4

\* The K excretion is controlled like that of the other electrolytes, by tubular reabsorption. In certain conditions K may be eliminated by tubular secretion too (27).

the anterior and posterior pituitary glands with their secreted hormones, and the endocrine glands they regulate; so we shall be concerned with the adreno-corticotrophic hormone (ACTH) and the separate hormones of the adrenals, and one should talk about their homeostatic action on the electrolytes, taking into consideration their point of action besides the results of synergism and antagonism by other hormones (25, 26). But I shall try and limit this discussion to the clinical action of these hormones on water and electrolyte metabolism under controlled conditions without going into unnecessary detail concerning the many hypotheses about points of attack and mechanisms of action.

It might be emphasized here, that these actions can be extensively varied both qualitatively and, even more so, quantitatively. They are dependent on dose, manner, and duration of administration, simultaneous intake of salt and water, and, of course, on the physiologic or pathologic state of the organism—to name only the most important factors.

#### CHEMISTRY OF HORMONES

Concerning the chemistry and occurrence of the adrenal cortical hormones, Fig. 2 shows the six steroids active in this respect which have been known since the 1930s, and to the isolation and structure of which a number of American and Swiss groups have contributed (6-8). The activity is closely linked to the presence of the  $\Delta^4$ -3-keto group and the reducing  $\alpha$ -ketol group in the  $17\beta$  position of the pregnene skeleton. Additional hydroxyl or keto groups in the  $11\beta$  or  $17\alpha$  position produce abundant variations of the biologic activity. A noncrystalline, nonhomogeneous fraction from the residue, the so-called "amorphous fraction," showed an especially powerful electrolyte activity. Out of this fraction our Anglo-Swiss team was able to isolate a new type of

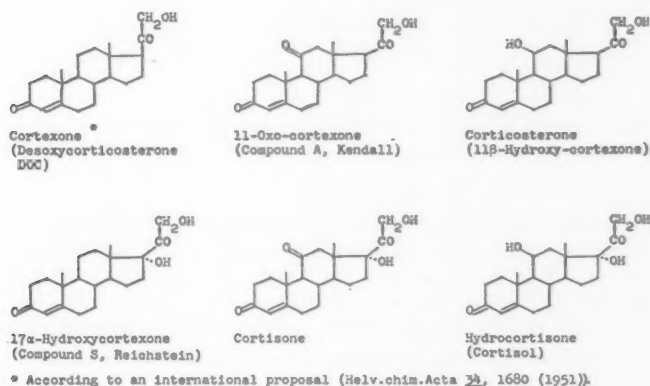
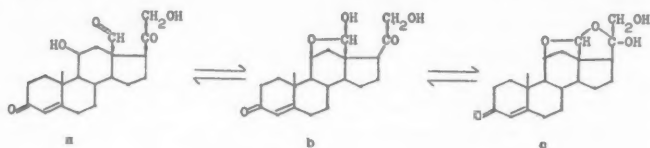


Figure 2

highly active steroid, aldosterone, in crystalline form (10, 10a; Fig. 3), and shortly afterward to elucidate the constitution (28), which was then verified by the groups of Sarett and Mattox (29, 30). This substance is the first example of a cortical steroid oxygenated in the 18 position, being an 18-oxo-corticosterone, existing in solution mostly as the forms (b) or (c).



Aldosterone

Figure 3

Very recently we have been able to isolate several other  $\Delta^4$ -3-ketopregnene derivatives with  $\alpha$ -ketol side chains from adrenals (31), these being additionally oxygenated in the  $6\beta$  or  $19$  positions besides occasionally having  $11\beta$  or  $17\alpha$  hydroxy groups. Figure 4 will give a general idea of the positions that can be occupied alone or in combination with hydroxyl or keto groups in pregnene derivatives isolated from adrenals.

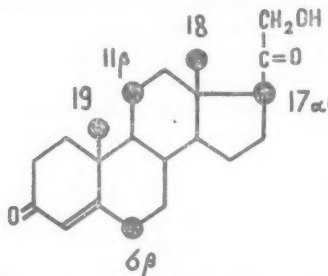


Fig. 4. Positions in  $\Delta^4$ -3-ketopregnene derivatives isolated from adrenals, and containing an  $\alpha$ -ketol side chain, that may be occupied, singly or in combination, with hydroxyl or keto groups.

Today the question about which of these substances are essential hormones in the adrenal cortex *in man* seems to be just about solved. Aldosterone, corticosterone, and hydrocortisone certainly belong to the "genuine" class, and have been detected in the adrenals (32-34), adrenal venous blood, peripheral blood (32, 35-39, 39a), and urine (40-45), and one might add the two substances 11-dehydrocorticosterone and cortisone, closely linked with corticosterone and hydrocortisone (37). Reichstein's substance S has been detected in human adrenals (31), although not in blood. The occurrence of cortexone in animal

adrenals (6, 46) and adrenal venous blood (47, 47a) has been established, but has yet to be demonstrated in humans; from a physiologic point of view, it can probably best be considered as serving as an intermediate in the formation of corticosterone (45) and aldosterone (49), but in any case its clinical usefulness is unquestionable (50). It is quite possible that more hormones will come to light in the future (152).

#### ACTION OF HORMONES

The adrenal cortical hormones are known to possess many kinds of biologic activity, from which we shall only deal here with what concerns the mineral metabolism. The classic division into mineralocorticosteroids and glucocorticosteroids seems, since the discovery of aldosterone and the synthetic adrenal hormone analogs, to be justified only from a quantitative point of view, since the separate qualifications of activity are greatly overlapping. I should like to use a classification based on the relative extent of the Na retention, having the added advantage that this is, together with the Na:K ratio, of paramount importance for the maintenance of life (4). From the results in a test on adrenalectomized rats worked out by Desaulles and Meier (51), we can see (Table 2) that the most active of the natural products is aldosterone, followed by cortexone, substance S, corticosterone, 11-dehydrocorticosterone, and, finally, hydrocortisone and cortisone, the activity of the last compared with aldosterone being in this test  $10^5$  times lower, measured by the Na retention, and  $2 \times 10^3$  by the Na:K ratio.

The first thing to do on studying the action of adrenal hormones on electrolyte and water metabolism is to make sure that the renal functioning is nor-

Table 2

Relative Activity of Adrenal Cortical Hormones on the Electrolyte and Water Metabolism of Adrenalectomized Rats<sup>a</sup>.

	Na Retention E.D. 50 mg./kg.	K Excretion E.D. 50 mg./kg.	Water Mobilization 30% Change mg./kg.	Na/K Ratio
Aldosterone	0.001	0.04	-	100
Cortexone	0.025	0.2	0.006 Ret.	1
17 $\alpha$ -Hydroxy- cortexone ("S")	0.8	13	6.5 Ret.	0.08
Corticosterone	5.5	1.3	40 Exor.	0.14
11-Dehydrocorticosterone	8.5	2.0	20 Exor.	0.07
Hydrocortisone	75	0.85	(1.3 Exor. >100 Ret.)	0.08
Cortisone	300	1.4	(15 Exor. >100 Ret.)	0.06

<sup>a</sup>) P. Desaulles and R. Meier (51)

mal; furthermore, a clear distinction must be made between the action in a normal organism and one with an adrenal deficiency. In the first case we shall be concerned with an overdosage effect, that is to say a *pharmacologic* activity, and in the case of adrenal deficiency (at the appropriate dose), with a specifically *physiologic* activity.

Let us next examine how the electrolyte metabolism of a human is influenced by loss of the adrenals, such as happens in Addison's disease (3) or by bilateral adrenalectomy (52). An immediate rise in the renal excretion of Na, Cl, and water takes place, with K retained, as a result of which dehydration, hemoconcentration, and lowering of the Na and Cl levels, with raising the K level in the serum occur. An acute case is illustrated by the serum ionogram in Fig. 5 (3). Mostly, in fact, the changes in the serum are not so marked, and also depend on whether the insufficiency has developed gradually or is of an acute nature. The extracellular loss of salt and water is greater than that explained by the renal excretion, so that in consequence a shift must have taken place from the extracellular to the intracellular space (52, 53). Similar changes can be found in the Na and K content of extrarenal excretions like sweat (54), saliva (55), or gastrointestinal secretions (56). If suitable doses of adrenal extracts or adrenal hormones are now given, a normalization of the electrolyte and water balance occurs, the adrenal hormones serving to maintain the homeostasis by retention of Na, Cl, and water, and elimination of K, in that they influence the renal and extrarenal electrolyte excretion, as well as the relative electrolyte distribution between extracellular and intracellular spaces (4).

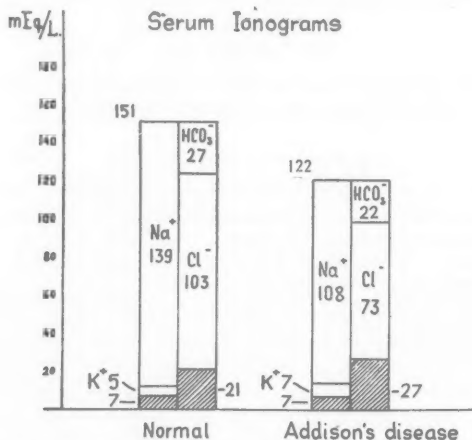


Fig. 5. Serum ionogram of a patient with acute Addison's disease (3) compared with that of a normal subject.



We should now analyze the action of the individual hormones on the overall balance of water, Na, K, and Cl, and, so far as they are of any interest, Ca, P, and Mg too, in humans with normal, and those with deficient adrenal functioning. I might say here that to get noticeable effects in normal people requires greater amounts of the hormones than are needed for Addisonians.

### CORTEXONE

On both historical and practical grounds we ought to begin with cortexone (desoxycorticosterone, DOC, desoxycorticosterone acetate, DCA) (50, 56-67); it may be given intramuscularly, subcutaneously or sublingually but is only slightly active when swallowed. Figure 6 illustrates the effect of cortexone acetate on the renal excretion of Na, Cl, K, and water of a patient with Addi-

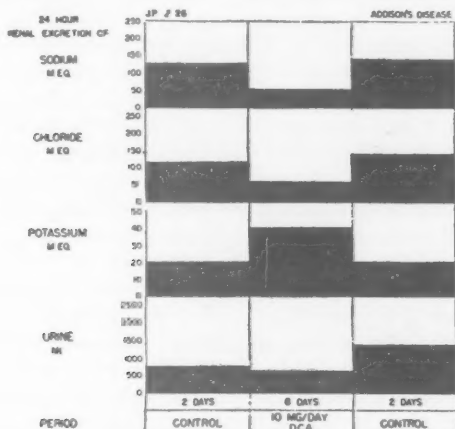


Fig. 6. Action of cortexone acetate on the renal excretion of Na, Cl, K, and urine in a patient with Addison's disease (67).

son's disease under normal salt intake (67). From this it can be seen that, measured against the average values for control treatment periods, a rapid normalization of the disturbed electrolyte balance occurs, that is to say a marked decrease of the Na, Cl, and water excretion, combined with an increase of K excretion. Ca and P levels do not appear to be affected uniformly. It is possible that water retention is the resultant of two antagonistic effects, a diuretic one, and an osmotic water-retaining one, the latter being conditioned by the Na retention (25). At the same time, an increase of the extracellular fluid occurs, especially of the plasma volume, and a displacement from the intracellular to the extracellular space is possible (3). The electrolyte displacements are obvious in the serum too, where Na and Cl levels rise and the K falls.

Hormone overdosage can be made to displace the serum electrolytes even beyond normal, to an extent comparable to that met with in adrenal hyperfunction. The cortexone treatment may be checked clinically by following the Na:K ratio, this corresponding better to the degree of neuromuscular disturbance than the individual values do (67). The identical action on the electrolyte relationships may be produced with the slow-acting trimethylacetate of cortexone (56, 68), for which a single injection of 30-60 mg. is enough for 5-6 weeks. If Addisonian patients are treated for a long time with high doses of cortexone, they can be brought to a state resembling that of diabetes insipidus. The large urine volume they produce seems to be largely the result of polydipsia brought on by excessive salt retention (26).

For Addisonian patients in general, doses of 1-5 mg. cortexone intramuscularly daily are sufficient to bring about an effect. Ten times as much is required by normal people, in whom, once again, within 2-4 hours, retention of Na, Cl, and water, accompanied by increased K excretion sets in (56, 61), but only if the salt intake is normal or raised. The Na retention preponderates from a quantitative point of view over the K loss, the latter not usually being accompanied by a P loss. Corresponding alterations in the serum of normal subjects too can be caused by cortexone. The Na and Cl retention is closely connected to there being a sufficient Na intake, although it does not matter whether the Cl intake is high or low (58, 69). Conversely, with restriction of Na, the Na and Cl retaining action of cortexone, as well as the K eliminating action will be lessened or even completely abolished (65, 66). These results would seem to imply that the K excretion brought about by cortexone is determined by the extent of the simultaneous Na retention (57).

In healthy people after a long duration of cortexone administration, an escape often occurs. Retention of the Na and Cl, and loss of K are suppressed, when the Na balance normalizes itself, followed by the K balance, so that finally it is possible to carry it to such an extent that a diabetes insipidus-like state occurs, in which the over-all balance of Na and Cl is negative (59, 60, 63, 65). Figure 7 shows the electrolyte balance and serum values of three cases receiving 40 mg. cortexone acetate daily under conditions of normal salt intake (63). First of all, Na, Cl, and water are retained and K is lost, Na being more strongly retained than Cl. Then a rebound phenomenon occurs, the original retention being replaced by an increased excretion, and a greater amount of Ca in the urine can be observed, as well as a rise of exchangeable and intracellular Na, in spite of a negative Na balance. With reduction of the salt supply to a maximum of 10 mEq./day, in contrast to the last experiment, no significant change in the Na, Cl, and K balances was observed and the serum electrolytes remained unaltered, as did the Ca excretion in the urine, although the Ca balance became negative.

To sum up, we can say that the physiologic action of cortexone consists to a large extent in Na retention, followed by Cl and water retention, and K loss.

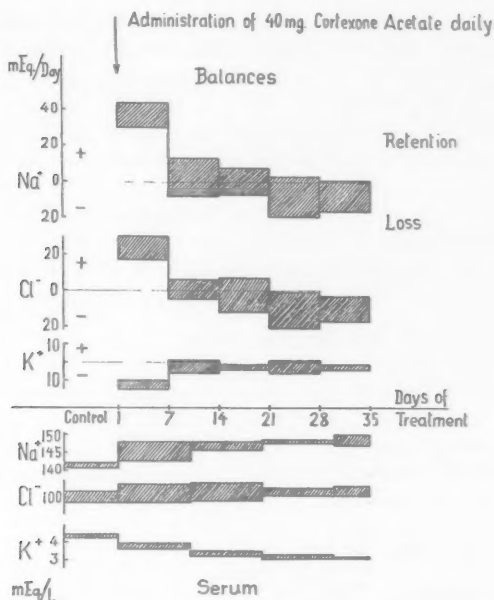


Fig. 7. Balances and serum concentrations of Na, Cl, and K in normal subjects during the administration of 40 mg. cortexone acetate daily over 5 weeks (12). The shaded areas indicate the ranges found in three subjects.

The changes in the Cl and K balance can be looked upon as consequences of the Na retention, although the water retention is certainly not dependent on the Na retention alone (50). Cortexone might in addition control the water and electrolyte distribution between the cells and interstitial fluid without intervention of the kidneys, and in extreme conditions of hormone dosage and salt and water intake it can lead to a diuresis.

### ALDOSTERONE

As a comparison with cortexone, we might consider aldosterone, up till now the most active of all the "genuine" electrolyte-regulating hormones known. Here we do not yet have detailed experiments to hand as we did with cortexone. Up to the present, nearly all investigations with aldosterone have had to be carried out with the natural product, the aldosterone being isolated by a very tedious process from frozen beef or hog adrenals in a yield of about 60-120 mg. per ton. Since Wettstein and his associates have managed to synthesize aldosterone (70, 71), the production of larger amounts for clinics is expected

in the near future, but even so, some preliminary clinical reports are able to give a good general idea of the action of aldosterone (39, 39a, 72-82, 151).

At first sight, looking at the electrolyte activity in a purely qualitative way, aldosterone appears to be very like cortexone in human subjects, although surpassing it quantitatively by many times. Addisonian patients can be brought to a normal state with as little as 100-300  $\mu$ g. aldosterone, which corresponds to a 20-30 times higher activity than that of cortexone. In short-term experiments aldosterone has proved to be about 40 times more active in Addison patients, and 60-100 times more in normal subjects (74). Aldosterone activity sets in rapidly and lasts for about 8 hours in doses of 100-300  $\mu$ g. Figure 8 shows a comparison between the activities of cortexone and aldosterone on the electro-

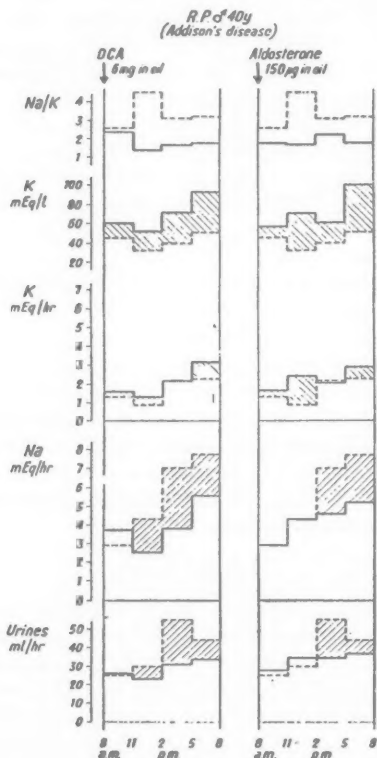


Fig. 8. Comparison of electrolyte effect between cortexone and aldosterone, on 3-hour urine samples, in a patient with Addison's disease (74).

lyte excretion in a short-term experiment. The dotted lines represent the values during the control periods, and the continuous lines those of the periods of treatment; the activity is shown up by the shaded areas (74). With a molecular proportion of dose of 1 aldosterone to 40 cortisone, about equally powerful Na, Cl, and water-retention and K excretion are obtained. The Na:K ratios, shown in Fig. 8, differ greatly from those of the control period. In an experiment of longer duration (80), an addisonian patient who had been brought out of the electrolyte equilibrium after withdrawal of cortisone therapy, responded well to treatment with only 100  $\mu$ g. daily for 9 days, during which time the electrolyte balance was restored. The Na serum values showed no particular change at this dose, the K level fell slightly. Water was retained to a small extent, and the extracellular volume increased somewhat. After stopping the treatment, it was still possible to observe a persisting positive balance of Na, Cl, and K. The Ca and P balances were not significantly changed. Doses of up to 1 mg. aldosterone daily led to correspondingly stronger effects.

The action in normal people is qualitatively the same. On the left hand side of Fig. 9 is shown the influence of 600–1000  $\mu$ g. aldosterone daily on the Na, Cl, and K excretion (81). During the period of treatment, the Na balance, only

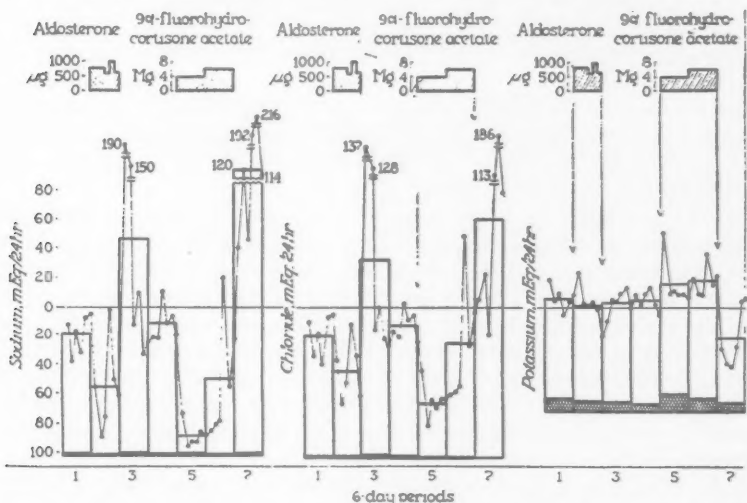


Fig. 9. Effect of aldosterone (left-hand side) on the excretion of Na, Cl, and K in a patient with normal adrenals (81). In this figure and Fig. 12 the daily intake is charted from the 0 line downward and the average daily excretion (feces below, urine above) from the bottom line upward. A negative balance is therefore indicated by extension of the column above the 0 line, and a positive balance, by a clear area below the 0 line.

slightly positive to start with, became quite distinctly positive, but cessation of the treatment caused Na loss and a transitory negative balance in the 6-day period following. Cl was retained to about the same extent as Na, as against K, the balance of which was not significantly affected. In the serum, neither Na, Cl, K, Ca, nor P were changed.

The influence of salt intake on electrolyte activity is also quite unmistakable in the case of aldosterone. Whereas a normal person with high salt intake reacts as described in the example just given, with salt restriction a normal person only shows an increased K excretion, without change of the Na and Cl balance (74). Up to the present, we have no detailed experiments of long duration with high doses of aldosterone concerning the influence of salt and water loads, though results with animals (83), and the fact that aldosterone excretion in urine is known to be dependent on the supply of various salts (84-86) make it likely that these factors do have a certain influence. The situation with respect to aldosterone at the moment is, therefore, that it is a highly active, specifically Na-retaining hormone, and, compared with cortexone, seems to cause K excretion in a somewhat different way, and to affect the water metabolism relatively less strongly.

### CORTISONE AND HYDROCORTISONE

As against the adrenal hormones that are primarily Na-retaining, there exists another group, influencing the electrolytes only relatively weakly, but having a high activity on the organic metabolism. Cortisone and hydrocortisone, the so-called "glucocorticosteroids," belong here, and may be dealt with together, since they are similar in both their qualitative action and their chemical constitution. Once again, only the electrolyte and water metabolism will be treated (50, 56, 63, 67, 87-112).

At the very beginning it was supposed, on the basis of animal experiments, that cortisone exerted an opposite or even antagonistic effect to cortexone on the Na excretion (113). A more exact study of the clinical effects later showed that the cortisone-hydrocortisone group influences the Na metabolism in qualitatively the same way under the same conditions as cortexone does, in other words, it can produce a retention. In contrast to cortexone, however, cortisone and hydrocortisone possess a relatively weak effect on Na in the usual therapeutic doses, combined with a diuretic action. It is therefore easy to understand that their influence on electrolyte metabolism is much more variable than that of cortexone or aldosterone (96, 104), and that it is especially dependent on the duration and the other conditions of the application. Besides the distinction between physiologic and pharmacologic activity according to dose, one must also bear in mind the action produced after treatment is stopped, because since these hormones show an inhibitory effect on the adrenocortical function, their withdrawal can result in the appearance of adrenocortical insufficiency.

Quantitatively, cortisone and hydrocortisone may be clearly distinguished

from each other, the strength of action being dependent on the method of administration (100, 101, 107, 109, 111, 112).

The Na-retaining action of cortisone acetate in Addisonian patients by intramuscular administration is only about 1/30-1/50 of that of cortisone acetate (93), as Fig. 10 shows, while the difference by intravenous infusion is a good deal less (50, 108). Besides the clear Na and Cl retention, cortisone acetate leads to an initial K loss, that is reduced step by step, so that in this way a positive K balance is often arrived at again, probably caused—partially, anyway—by the powerful action of cortisone on the carbohydrate metabolism, and the K binding associated with this (50, 67, 93). This effect on the K balance has recently been the subject of more thorough studies (87, 97), during which

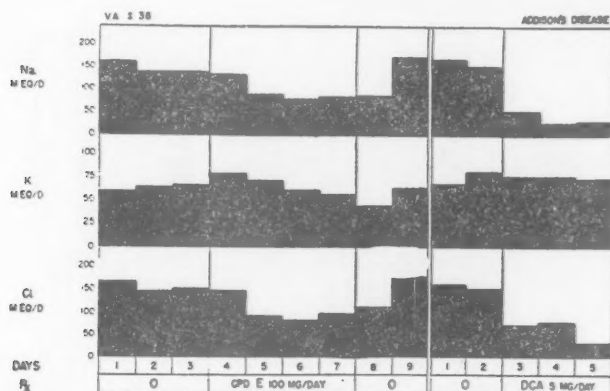


Fig. 10. Comparison of the effects of cortisone acetate (CPD E) and cortisone acetate (DCA) on urinary electrolyte excretion at constant diet in a patient with Addison's disease (93).

it was confirmed that, in patients with Addison's disease and in normal subjects, the influence of cortisone on the Na and K excretion differs both in time and degree. By intravenous infusion of 50-100 mg. hydrocortisone over half an hour K loss already reaches its maximal value after 4-6 hours, while Na and Cl excretion is still unchanged. This only starts later, and persists for 24 hours, during which time K has become retained again. The potassium level in the serum rises sharply in the first 4 hours, despite the loss in the urine; Na and Cl remain unaltered. The K level only falls later on, so that the K elimination from the cells must take place quicker than excretion via the kidneys. For this reason the early onset of the main effect probably is to be found in the cation distribution between the intracellular and extracellular fluid.

Thorn and his colleagues made an interesting short-term experiment with

hydrocortisone (109), from which as an example Fig. 11 illustrates the differences with dosage alterations. Hydrocortisone, administered intravenously at a rate of 10 mg. per hour (shaded areas) causes a preliminary Na excretion of short duration before the Na retention, both in addisonian and normal subjects. If the rate is decreased to 1 mg. per hour (black areas), the initial Na diuresis in addisonian patients lasts a great deal longer, over 24 hours, in fact. The Na diuresis seemed to be the result of an increase in the glomerular filtration rate, this being larger than the tubular reabsorption, while the Na retention that rapidly succeeds this when higher doses of hydrocortisone are given was accounted for by increase of the tubular reabsorption.

The plasma values are on the whole only slightly changed. The abnormal distribution between extracellular and intracellular water occurring in adrenal

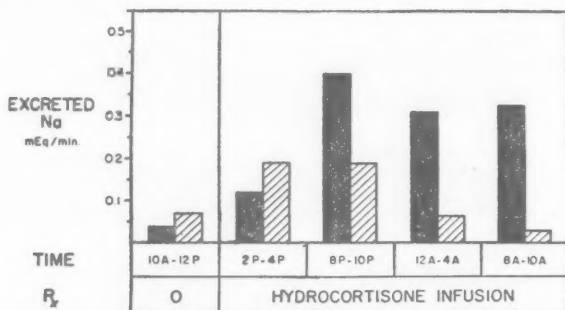


Fig. 11. Effect of continuous intravenous hydrocortisone on the excretion of Na in Addison's disease (109). Black area: hydrocortisone 1 mg./hr. Shaded area: hydrocortisone 10 mg./hr.

insufficiency and the intoxication resulting from a water load are normalized, or corrected by facilitation of the diuresis, in contrast to cortisone (50, 56, 93).

The activities mentioned are quantitatively the same in normal people (89). Figure 12 shows the way long-term experiments turn out (96, 100, 104). While the effects of 100 mg. cortisone acetate intramuscularly are very small, they become much more important when 200 mg. per day intramuscularly are given, as we shall see from the example (96) of a patient who had intact kidneys and adrenals. Treatment with cortisone produced a fall in plasma Cl and K, but Na showed a tendency to rise. In the first two periods of the treatment the Na and Cl retention may be clearly seen, but in the third period, and especially in the period immediately after, the Na and Cl balances became negative. Only in the third period of treatment did the K balance become negative. Ca and P excretion in the feces was variable, but on the whole increasing, especially in the first posttreatment period, with a slightly increased renal Ca and P excre-



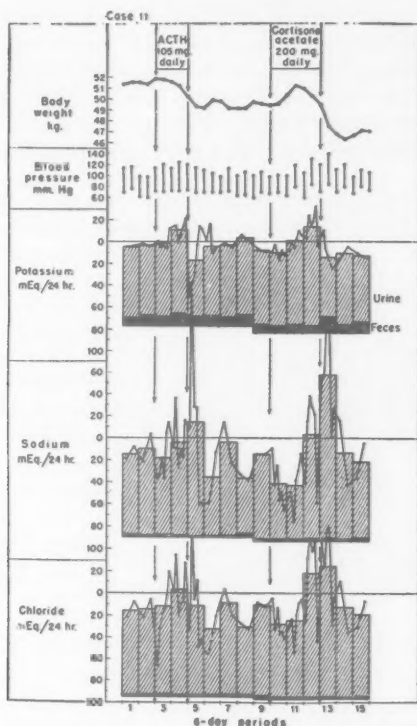


Fig. 12. Effect of long-term treatment (intramuscularly) with 200 mg. cortisone acetate daily on the balances of Na, Cl, and K in a patient with normal adrenals (96).

tion during the treatment (96, 99). Long-term treatment with cortisone can lead to hyponatremia, hypokalemia, and osteoporosis, in other words, to similar symptoms as in Cushing's syndrome.

A few authors have studied the influence of salt intake on the action of cortisone in long-term experiments (63, 94). As opposed to experiments with a normal salt intake, the ones we have just been discussing, the Na and Cl balance under salt restriction is only altered a little, with a slight tendency towards a negative balance. K, Ca, and P balances, on the other hand, are negative, as with normal salt intake. In both cases, K in the plasma is lessened, but Na and Cl are unchanged.

The influence of cortisone on the water metabolism in the body may be caused not only through the electrolytes, but also inhibition of the release of the antidiuretic hormone of the neurohypophysis (144) and through a displace-

ment of the intracellular and extracellular fluid distribution (50, 96, 99, 103, 110). Transient shifts of water, Na, and Cl into the extracellular space occur, with reduction of the intracellular water, but without change of the total body water. Furthermore, changes in the renal function by raising the glomerular filtration rate and renal plasma flow have been found (96, 103). The normal daily rhythm of water and electrolyte excretion is masked or suppressed by cortisone (95).

We can summarize all these facts about cortisone and hydrocortisone in the following way: in general, they are able to produce as physiologic reactions Na and Cl retention and increase of K excretion, with the reservation that these actions are highly dependent on various factors, and the resultant effect may often be completely reversed. There is no evidence for an influence on fecal Na, Cl, or K (92). Although no significant change in the serum Ca and P is observable, the balance is displaced to the negative side because of renal and fecal losses, with possible causing of osteoporosis (88, 99, 106). Magnesium is generally little influenced, probably only through the changes in the Cl excretion (114); occasionally a slight retention has been observed (102).

We have left out of the list of adrenal cortical hormones so far described corticosterone, 11-dehydrocorticosterone, and Reichstein's substance S. These steroids, among which at least corticosterone can be counted as one of the "genuine" adrenal hormones, may be put together in a third group, whose action on the electrolyte metabolism is roughly between that of the first and second groups.

### CORTICOSTERONE AND 11-DEHYDROCORTICOSTERONE

Corticosterone and 11-dehydrocorticosterone are outstanding neither as mineralocorticoids nor as glucocorticoids. They influence mineral metabolism to a rather greater extent than organic metabolism. The activities of both compounds on the electrolyte metabolism that are apparent clinically (50, 56, 67, 109, 115, 116) are very similar, and can be briefly treated together. They correspond on the whole to those of cortexone, but are about 25-50 times weaker by intramuscular administration, being, however, a bit stronger than those of cortisone. The 11 $\beta$ -hydroxy compound seems to be more active than the 11-keto compound, though there are no exact studies available for different methods of application. Corticosterone is 2-3 times more active orally than parenterally. Addison patients can be brought to a normal condition with 25-50 mg. of corticosterone per day intramuscularly or 40-100 mg. of 11-dehydrocorticosterone, by retention of Na, Cl, and water, and a moderate increase in the excretion of K. These steroids are also able, to a certain degree, to protect the patients against water load. In normal subjects one can also see, using a longer corticosterone treatment, the same phenomena as with the other adrenal hormones mentioned before, escape of the retained Na and Cl and decreased K excretion. At times, an increase of P excretion has also been observed.

## SUBSTANCE "S"

The degree of activity of Reichstein's substance *S* on the electrolytes is so low that it can hardly be considered even as a hormone. Clinically, it is parenterally and orally active in 50-100 times higher dosage than cortexone (56, 117, 118). In adrenalectomized or normal subjects, a slight retention of Na and Cl may be demonstrated, but only with at least 200-300 mg. daily administered for several days, while the metabolism of K, Ca, P, and water seems to remain uninfluenced. Just as little change in the serum is observed.

## ACTH

This analysis of the individual, naturally occurring adrenal hormones known up to now has shown them to differ from one another, above all quantitatively, in their influence on electrolyte metabolism under fixed conditions. It will certainly be interesting to have a look now at the action of the endogenic hormone mixture put out by the adrenal cortex to an increased extent under stimulation by exogenic ACTH. Qualitatively, this mixture is most likely different from the usual adrenal cortex secretion, since it seems that ACTH stimulates the production of hydrocortisone and corticosterone preferentially, and that of aldosterone only indirectly and to a much lesser extent (39, 146-150). The clinical effects of exogenic ACTH in people with a normal adrenal function (50, 56, 63, 67, 91, 92, 96, 99, 103, 104, 108, 110, 119-121) similarly manifest themselves, especially in brief experiments, by a retention of Na, Cl, and water, and a definite K loss, just the same as we have already seen with the adrenal cortical hormones. In long-term studies, for instance with 100 mg. of ACTH daily (96), more or less sharp changes of the electrolytes in the plasma level become apparent; Na, Cl, and K decrease, and a hypochloremic, hypokalemic alkalosis develops. Nevertheless, one can observe an initial retention of Na and Cl from the balance, although this becomes negative again in the later periods of treatment, and even more strongly so after stopping the ACTH treatment, being followed once again by a rebound with renewed Na and Cl retention. K is fairly strongly excreted during the treatment, and retained again afterwards. No effect on fecal Na, K, and Cl can be seen (92), and excretion of P and Ca in the urine and feces is not uniform with ACTH (120). Although osteoporosis is occasionally observed with ACTH (88), the distribution of Ca between plasma and bones does not seem to be influenced (121). With an excessive treatment of ACTH, a Cushing-like state can arise as it does with cortisone (50). Moreover, changes in the glomerular filtration rate are observed with ACTH, as well as in the renal plasma flow (103, 110), and, as extrarenal effects, transient shifts of water and electrolytes into the extracellular space (94, 110) and within the tissue take place (99). In contrast to the regular appearance of these effects with normal salt intake, there are several that disappear with restriction of salt or increase of K intake (63, 119, 122). In par-

ticular, the Na and Cl retention is diminished by this, or even made to completely disappear, in spite of which the K balance becomes strongly negative. On withdrawal of K, the usual Na retention persists just as strongly.

Figure 13 (105) summarizes the effect on normal subjects of intravenously administered ACTH and adrenal hormones, except aldosterone whose activity is—under the conditions given—for the most part only quantitatively different from that of cortexone. Na retention of the compounds mentioned is exhibited above all by cortexone and ACTH, immediately followed by hydrocortisone, and finally by corticosterone. The 2 hours' delay before the onset of ACTH

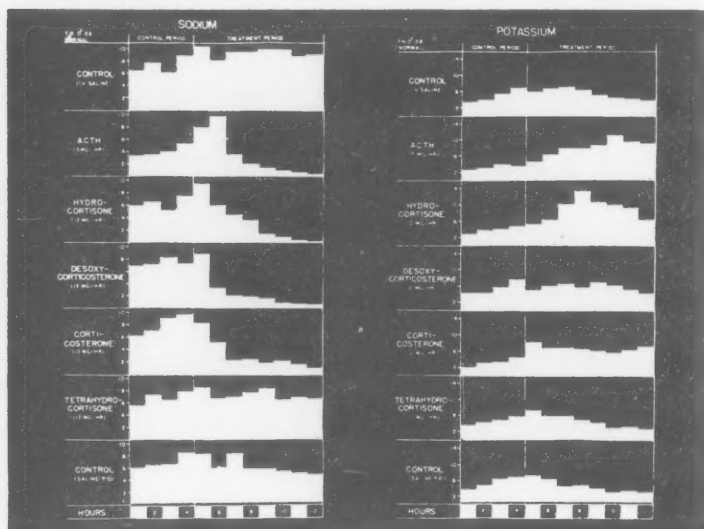


Fig. 13. Comparison of effect of intravenous ACTH and adrenal cortical hormones (with exception of aldosterone) on urinary Na and K excretion. Units of ordinates: mEq./hr. (105).

action is remarkable when compared with the 1 hour of the other hormones; the difference is probably due to the time lag required by ACTH for the stimulation of the adrenal cortex. With the same conditions, the K excretion is significantly raised with ACTH and hydrocortisone infusion, and less with cortexone and corticosterone. It seems quite possible, therefore, that the changes in electrolyte excretion caused by exogenic ACTH owe at least part of their occurrence to the induced secretion of the corresponding amount of hydrocortisone.

## ADRENAL HYPERFUNCTIONING

We have now followed how electrolyte changes are caused by adrenal insufficiency, and how they are influenced through intake of adrenal hormones by subjects with or without functionally fit adrenals. In order to round off the picture, it still remains to examine how hyperfunctioning of the adrenals, that is to say, an endogenic overdosage of single groups of adrenal hormones, affects the electrolyte metabolism. To do this, let us consider two syndromes associated with a different type of adrenal hyperfunctioning, namely Cushing's syndrome, with an exceptionally high production of hydrocortisone and cortisone, and Conn's syndrome, caused primarily by overproduction of aldosterone. The corticosteroid content of human adrenal tissue in such cases is shown in Table 3 (34). While the adrenal hyperplasia given in this example, seems, in fact, to be the result of an augmented production of *all* hormones, which does not amount to anything in terms of  $\mu\text{g./Gm.}$ , the proportional pro-

Table 3  
Cortical Hormones in Adrenal Tissue (34)

	$\mu\text{g./g.}$		
	Adrenal Hyperplasia	Conn's Syndrome (Tumor)	Cushing's Syndrome (Tumor)
Aldosterone	0.25	1.4	0.28
Hydrocortisone } Cortisone }	5.65	9.0	39.0
Corticosterone	3.5	32.5	< 0.8

duction of hormones in Cushing's syndrome and especially in primary aldosteronism is very different. With *Cushing's syndrome* (123-126) the aldosterone production, measured in the adrenal tissue or by the excretion in the urine (84), is only slightly raised, as against the more pronounced increase in the production of hydrocortisone and cortisone.

This situation, in terms of the electrolyte metabolism, does not produce any very marked displacements. The serum values are, for the most part, normal, as are those for Ca and P, despite some demineralization. If it does come to a change, then it is first of all to a negative K balance. On the other hand, very pronounced effects are observed in *primary aldosteronism*, which used to be classed under cases of potassium-losing nephritis (127, 128). The 5-30 times higher aldosterone production as estimated from the adrenal tumor content observed here, is, with the high activity of aldosterone, more than enough to produce marked electrolyte displacements like hypokalemia, hypomagnesemia, increase of K and Mg excretion, and retention of Na. A raised aldosterone excretion in the urine is correspondingly observed (84). In the case of the

adrenal tumor we studied, besides aldosterone, corticosterone was also raised considerably (34), which might contribute additionally to the disturbance of the electrolyte metabolism. The latter was normalized on removal of the tumor. From this example, it is clearly shown that the action of exogenic and endogenic overdosage is identical.

### ELECTROLYTES IN SWEAT AND SALIVA

At the beginning, I mentioned that the adrenal cortical hormones possess extrarenal activity on the electrolyte metabolism too. Quite apart from the influence on the gastrointestinal tract (56, 129, 130) and the extracellular and intracellular space, we might think of the action on the sweat and saliva secretions that are used for clinical diagnosis. Conn (54) has observed that the Na and Cl concentration in *sweat* is largely controlled by adrenal activity. While, for instance, ACTH (131) or cortexone (56, 132) produce an unmistakable fall in Na and Cl in the sweat, adrenalectomy acts in precisely the opposite way. Overproduction of endogenic aldosterone in cases of Conn's syndrome (128) also effects a lowering of the Na concentration, but this has not yet been clearly observed with exogenic aldosterone (73). Following the Na changes in sweat has the advantage, that, in contrast to urine, no escape seems to occur in course of treatment. A disadvantage is that there is a certain amount of difficulty in collecting thermal sweat under constant conditions.

Analogous effects take place in the *saliva*, for which Frawley and Thorn (55) recommended expressing the electrolyte concentration in the form of the Na:K ratio for an estimation of the adrenal function. A drop in the Na:K ratio has been observed in Cushing's syndrome, with endogenic and exogenic aldosterone (39, 73, 128), cortexone, corticosterone (56), cortisone (133), and ACTH (134), and a rise in Addison's disease and adrenalectomy (55). More exact studies have shown that K is not influenced, and that stimulation of the saliva flow by chewing paraffin or with pilocarpine leads to the identical result (133, 135). On the other hand, the values according to age, daily variation, duration of stimulus, and other factors seem to vary quite a lot, so that the reliability in many cases appears to be questionable. For instance, in patients with nephrotic syndrome, or with operative trauma, no changes in sweat could be observed (133), that is to say, in cases where a raised aldosterone excretion in the urine can be easily detected (40-42, 84). Taken in conjunction with other clinical tests, these methods can without doubt, however, provide a useful service.

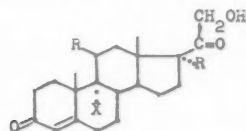
### SYNTHETIC ANALOGS

Up till now, we have been exclusively concerned with the activity of the naturally occurring adrenal cortical hormones, so now we still have finally to take into account the action of *synthetic analogs* that have recently become important clinically. Highly active derivatives have been obtained lately, especially of hydrocortisone (cortisol), by introduction of halogen in the 9 $\alpha$ -

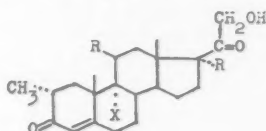
position (11), a methyl group in the 2 $\alpha$ -position (14), or an additional double bond in the 1,2-position (12), these modifications being alone or in combination (13 and Fig. 14). If we consider only the electrolyte metabolism, we can divide these substances into two groups. The first, comprising the 9 $\alpha$ -halogen and 2 $\alpha$ -methyl derivatives, exhibits a very strong increase of the Na-retaining properties of the parent substances, the other group, the 1-dehydrocompounds, causes a diminution of the Na retention. For example, 1-dehydrocortisol (prednisolone) administered either orally or intravenously shows, in clinical experiments (76, 136-138) on patients with both insufficient and normal adrenal functioning, at least a four times weaker Na retention, as compared with cortisol, the K excretion being hardly or not at all influenced. No changes were observed in the serum levels of Na, K, Cl, Ca, or P.

Among the 9 $\alpha$ -halogen derivatives (76, 109, 139, 142), 9 $\alpha$ -fluorocortisol especially proved to be at least 50 times stronger Na-retaining than cortisol when it was administered orally or intravenously to patients with or without intact adrenals, and even stronger than cortexone, in fact, almost as strong as aldosterone. Water retention and K loss are increased to about the same extent. At the same time the Na and K levels in the serum are markedly changed in opposite directions. If 9 $\alpha$ -fluorocortisol contains an additional 1,2 double bond (76), then the Na-retaining activity is only very slightly diminished (76, 145).

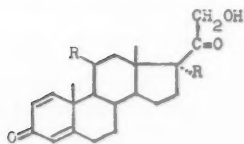
A very similar increase of the effect on electrolytes as the fluorine atom in the 9 $\alpha$ -position of cortisol is caused by a methyl group in the 2 $\alpha$ -position (143). If both the 2 $\alpha$ -methyl and 9 $\alpha$ -fluoro groups in cortisol are combined, a compound is obtained that is the strongest Na-retaining agent known today (143).



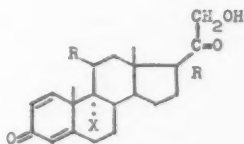
I R = H or OH  
X = F, Cl, Br



II R = H or OH  
X = H or F



III R = H or OH



IV R = H or OH

Fig. 14. Synthetic analogs of adrenal cortical hormones with substituents in the 2 $\alpha$ -position and/or a double bond in the 1,2- position.

## CONCLUSION

In summarizing, we can say that both the "genuine" and synthetic substances which are known at present and which exhibit adrenal cortical activity—in relation to the electrolyte metabolism, but not the water metabolism—all show qualitatively similar clinical activity under controlled conditions, but with wide quantitative differences. If the various influences like dosage, duration of treatment, and salt and water load are borne in mind, together with the correlated influence of the endocrine glands, it is not surprising that some of the individual activities may be finally reversed. Escape and rebound phenomena can be considered as compensatory effects. There is with most hormones, moreover, a strong action on the organic metabolism, which in turn has an indirect influence on the electrolyte metabolism. For an evaluation of the type of activity of a particular hormone, therefore, not only the absolute activity must be considered, but more especially, the various relative activities. There are certainly quite a number of hitherto undescribed substances in the adrenals. Whether these substances have any specific activity of another sort on the electrolyte metabolism must remain for the present an open question. There are some indications (152) that the present picture of the action of the adrenal cortical hormones on electrolyte and water metabolism will have to be modified in the near future.

## REFERENCES

1. Loeb, R. F., *Science* **76**, 420 (1933).
2. Loeb, R. F., *Proc. Soc. Exper. Biol. & Med.* **30**, 808 (1933).
3. Loeb, R. F., *Bull. New York Acad. Med.* **18**, 263 (1942).
4. Russell, J. A., and Wilhelmi, A. E., in Lukens, F. D. W., *Medical Uses of Cortisone*, New York, Blakiston, p. 1 (1954).
5. Osler, W., *Internat. M. Magazine* **5**, 3 (1896).
6. Reichstein, T., and Shoppee, C. W., *Vitamins and Hormones* **1**, 345 (1943).
7. Heard, R. D., in Pincus and Thimann, *The Hormones*, New York, Acad. Press (1948), p. 549.
8. Shoppee, C. W., and Shoppee, E., in Rodd, *Chemistry of Carbon Compounds*, Amsterdam, Netherlands, Elsevier, (1953), p. 929.
9. Wettstein, A., and Anner, G., *Experientia* **10**, 397 (1954).
10. Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., v. Euw, J., and Reichstein, T., *Experientia* **9**, 333 (1953).
- 10a. Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., v. Euw, J., Schindler, O., and Reichstein, T., *Helvet chim. acta*, **37**, 1163 (1954).
11. Fried, J., and Sabo, E. F., *J. Am. Chem. Soc.* **75**, 2273 (1953). **76**, 1455 (1954).
12. Herzog, H. L., et al., *Science* **121**, 176 (1955).
13. Hirschmann, R. F., Miller, R., Beyler, R. E., Sarett, L. H., and Tishler, M., *J. Am. Chem. Soc.* **77**, 3166 (1955). Fried, J., et al., *J. Am. Chem. Soc.* **77**, 4181. Nobile, A., et al., *J. Am. Chem. Soc.* **77**, 4184. Hogg, J. A., et al., *J. Am. Chem. Soc.* **77**, 4438. Vischer, E., Meystre, C., and Wettstein, A., *Helvet chim. acta* **38**, 835, 1503 (1955).
14. Hogg, J. A., Lincoln, F. H., Jackson, R. W., and Schneider, W. D., *J. Am. Chem. Soc.* **77**, 6401 (1955).
15. Manery, J. F., *Physiol. Rev.* **34**, 334 (1954).



16. Gamble, J. L., *Chemical Anatomy, Physiology and Pathology of Extracellular Fluid*. Harvard, Cambridge, Mass., 1949.
17. Weisberg, H. F., *Water, Electrolyte and Acid-Base Balance*. Baltimore, Williams & Wilkins, 1953.
18. Edelman, I. S., et al., *Surg. Gynec. & Obst.* **95**, 1 (1952). (Deuterium oxide method.)
19. Levitt, M. F., and Gaudino, M., *Am. J. Med.* **9**, 208 (1950). (Inulin method.)
20. Gibson, J. G., and Evans, W. A., *J. Clin. Invest.* **16**, 317 (1937). (Evans blue method.)
21. White, A. G., Gordon, H., and Leiter, L., *J. Clin. Invest.* **29**, 1445 (1950).
22. Ahlman, K. L., Eränkö, O., Karvonen, M. J., and Leppänen, V., *J. Clin. Endocrinol.* **13**, 773 (1953).
23. Mudge, G. H., *Bull. New York Acad. Med.* **29**, 846 (1953).
24. Bernard, C., *Leçons sur les phénomènes de la vie*. Paris, 1878.
25. Gaunt, R., Birnie, J. H., and Eversole, W. J., *Physiol. Rev.* **29**, 281 (1949).
26. Gaunt, R., and Birnie, J. H., *Hormones and Body Water*, Springfield, Ill., Thomas, 1951.
27. Berenson, G. S., and Burch, G. E., *J. Lab. Clin. Med.* **42**, 58 (1953).
28. Simpson, S. A., Tait, J. F., Wettstein, A., Neher, R., v. Euw, J., Schindler, O., and Reichstein, T., *Experientia* **10**, 132 (1954); *Helvet. chim. acta* **37**, 1200 (1954).
29. Mattox, V. B., Mason, H. L., and Albert, A., *Proc. Staff. Meet. Mayo Clin.* **28**, 569 (1953).
30. Harman, R. E., Ham, E. A., De Young, J. J., Brink, N. G., and Sarett, L. H., *J. Am. Chem. Soc.* **76**, 5035 (1954). Ham, E. A., et al., *J. Am. Chem. Soc.* **77**, 1637 (1955).
31. Neher, R., and Wettstein, A., *Helvet. chim. acta* **39**, 2062 (1956).
32. Hudson, P. B., and Lombardo, M. E., *J. Clin. Endocrinol.* **15**, 324 (1955).
33. Bloch, E., Benirschke, K., and Rosenberg, E., *Endocrinology* **58**, 626 (1956).
34. Neher, R., *Schweiz. med. Wchnschr.* **86**, 1262 (1956). Symposium on aldosterone, Geneva, 1957, in press.
35. Sweat, M. L., *J. Clin. Endocrinol.* **15**, 1043 (1955).
36. Eberlein, W. R., and Bongiovanni, A. M., *J. Clin. Endocrinol.* **15**, 1524 (1955).
37. Tamm, J., *Klin. Wchnschr.* **34**, 346 (1956).
38. Pincus, G., and Romanoff, E. B., *CIBA Found. Coll. Endocrinol.* **8**, 97 (1955).
39. Simpson, S. A., and Tait, J. F., *CIBA Found. Coll. Endocrinol.* **8**, 204 (1955).
- 39a. Simpson, S. A., and Tait, J. F., *Recent Progr. Hormone Res.* **11**, 183 (1955).
40. Luetscher, Jr., J. A., Neher, R., and Wettstein, A., *Experientia* **10**, 456 (1954).
- 40a. Luetscher, Jr., J. A., Dowdy, H., Harvey, J., Neher, R., and Wettstein, R., *J. Biol. Chem.* **217**, 505 (1955).
41. Luetscher, Jr., J. A., Neher, R., and Wettstein, A., *Experientia* **12**, 22 (1956).
42. Llauro, J. G., Neher, R., and Wettstein, A., *Clin. chim. acta.* **1**, 236 (1956).
43. de Courcey, C., Bush, I. E., Gray, C. H., and Lunnon, J. B., *Endocrinology* **9**, 401 (1953).
44. Cope, C. L., and Hurlock, B., *Clin. Sci.* **13**, 69 (1954).
45. Dorfman, R. I., and Ungar, F., *Metabolism of Steroid Hormones*. Minneapolis, Burgess, 1953.
46. Zaffaroni, A., and Burton, R. B., *J. Biol. Chem.* **193**, 749 (1951).
47. Jacobsen, R. P., and Pincus, G., *Am. J. Med.* **10**, 531 (1951).
- 47a. Farrell, G. L., Rauschkolb, E., and Royce, P., *Proc. Soc. Exper. Biol. & Med.* **87**, 587 (1954).
48. Haines, W. J., *Rec. Progr. Hormone Res.* **7**, 255 (1952).
49. Wettstein, A., Kahnt, F. W., and Neher, R., *CIBA Found. Coll. Endocrinol.* **8**, 170 (1955); Kahnt, F. W., Neher, R., and Wettstein, A., *Experientia* **11**, 446 (1955).

50. Thorn, G. W., *et al.*, in Lukens, F. D. W., *Medical Uses of Cortisone*. New York, Blakiston, 1954, p. 46.
51. Desaulles, P., and Meier, R. To be published.
52. Mendelsohn, M. L., and Pearson, O. H., *J. Clin. Endocrinol.* **15**, 409 (1955).
53. Hills, A. G., Chalmers, T. M., Webster, G. D., Jr., and Rosenthal, O., *J. Clin. Invest.* **32**, 1236 (1953).
54. Conn, J. W., *Arch. Int. Med.* **83**, 416 (1949).
55. Frawley, T. F., and Thorn, G. W., *Proc. Second Clin. ACTH Conf.* **1**, 115 (1951).
56. Thorn, G. W., *et al.*, *New England J. Med.* **248**, 232, 284, 323, 369, 414, 588, 632 (1953).
57. Esselier, A. F., Holtmeier, H. J., and Jeanneret, P., *Klin. Wchnschr.* **33**, 814 (1955).
58. Pulman, T. N., and McClure, W. W., *Metabolism* **3**, 240 (1954).
59. Luft, R., and Sjögren, B., *Metabolism* **2**, 313 (1953).
60. Luft, R., and Sjögren, B., *Acta endocrinol.* **10**, 49 (1952).
61. Fourman, P., *et al.*, *Metabolism* **1**, 242 (1953).
62. Warming-Larsen, A., and Sprechler, M., *Acta endocrinol.* **12**, 35 (1953).
63. Luft, R., Sjögren, B., Ikkos, D., Ljunggren, H., and Tarukosi, H., *Rec. Progr. Hormone Res.* **10**, 425 (1954).
64. Dauphinee, J. A., *Canad. J. Biochem. Physiol.* **33**, 493 (1955).
65. Belman, A. S., and Schwartz, W. B., *Yale J. Biol. & Med.* **24**, 540 (1952).
66. Luft, R., and Sjögren, B., *Stanford M. Bull.* **9**, 218 (1951).
67. Thorn, G. W., and Forsham, P. H., *Rec. Progr. Hormone Res.* **4**, 229 (1949).
68. Wieland, P., Heer, J., Schmidlin, J., and Miescher, K., *Helvet. chim. acta* **34**, 354 (1951).
69. Jeanneret, P., Esselier, A. F., and Holtmeier, H. J., *Helvet. med. acta* **23**, 60 (1956).
70. Schmidlin, J., Anner, G., Billeter, J. R., and Wettstein, A., *Experientia* **11**, 365 (1955).
71. Vischer, E., Schmidlin, J., and Wettstein, A., *Experientia* **12**, 50 (1956).
72. Prunty, F. T. G., *et al.*, *Lancet* **2**, 620 (1954).
73. Mach, R. S., *et al.*, *Schweiz. med. Wchnschr.* **84**, 407 (1954).
74. Muller, A. F., Mach, E., and Naegeli, A., *Acta endocrinol.* **20**, 113 (1955).
75. Hertz, B. S., *et al.*, *J. Endocrinol.* **13**, 112 (1956).
76. Thorn, G. W., Renold, A. E., Morse, W. I., Goldfien, A., and Reddy, W. J., *Ann. Int. Med.* **43**, 979 (1955).
77. Beck, J. C., Giroud, C. P., Dyrenfurth, I., and Venning, E. H., *Canad. J. Biochem. Physiol.* **33**, 884 (1955).
78. Mach, R. S., and Fabre, J., *CIBA Found. Coll. Endocrinol.* **8**, 361 (1955).
79. Kekwick, A., and Pawan, G. L. S., *Lancet* **2**, 162 (1954).
80. Griboff, S. L., Wiener, R., Eisenberg, J., Innacone, A., and Soffer, L. J., *Metabolism* **4**, 289 (1955).
81. Ward, L. E., Polley, H. F., Slocumb, Ch. H., Hench, Ph. S., Mason, H. L., Mattox, V. R., and Power, M. H., *Proc. Staff Meet. Mayo Clin.* **29**, 649 (1954).
82. Muller, A. F., and Engel, E., *Helvet. med. acta* **22**, 490 (1955).
83. Gaunt, R., Renzi, A. A., and Chart, J. J., *J. Clin. Endocrinol.* **15**, 621 (1955).
84. Wettstein, A., in *Lettré-Inhoffen-Tschesche's Handbuch: Ueber Sterine, Gallensäuren und verwandte Stoffe*. Vol. 2, Enke, Stuttgart, Germany. In press.
85. Luetscher, J. A., Jr., and Curtis, R. H., *Ann. Int. Med.* **43**, 658 (1955).
86. Falbriard, A., Muller, A. F., Neher, R., and Mach, R. S., *Schweiz. med. Wchnschr.* **85**, 1218 (1955).
87. Knight, R. P., *et al.*, *J. Clin. Endocrinol.* **15**, 176 (1955).
88. Fischer, F., and Hastrup, B., *Acta endocrinol.* **16**, 141 (1954).
89. Kowalski, H. J., *et al.*, *Proc. Soc. Exp. Biol. Med.* **83**, 795 (1953).

90. Fourman, P., Bartter, F. C., Albright, F., Dempsey, E., Carrole, E., and Alexander, J., *J. Clin. Invest.* **29**, 1462 (1950).
91. Sprague, R. G., *Am. J. Med.* **10**, 567 (1951).
92. Danowski, T. S., et al., *Proc. Soc. Exp. Biol. Med.* **81**, 445 (1952).
93. Thorn, G. W., et al., *Am. J. Med.* **10**, 595 (1951).
94. Walser, M., Seldin, D. W., and Burnett, C. H., *Am. J. Med.* **13**, 454 (1955).
95. Rosenbaum, J. D., Ferguson, B. C., Davis, R. K., and Rossmeisl, E. C., *J. Clin. Invest.* **31**, 507 (1952).
96. Sprague, R. G., et al., *Arch. Intern. Med.* **85**, 199 (1950).
97. Kelly, L. W., Levy, R. P., Sydnor, K. L., and Jeffries, W. Mak., *J. Lab. & Clin. Med.* **44**, 818 (1954).
98. Thorn, G. W., et al., *New England J. Med.* **245**, 549 (1951).
99. Eliel, L. D., Hellmann, L., Pearson, O. H., and Katz, B., *Proc. Second Clin. ACTH Conf.* **1**, 196 (1951).
100. Conn, J. W., Louis, L. H., and Fajans, S. S., *Science* **113**, 713 (1951).
101. Conn, J. W., et al., *J. Lab. & Clin. Med.* **38**, 799 (1951).
102. Bartter, F. C., et al., *Pituitary-Adrenal Function*. Washington, D. C., Amer. Assn. for the Advancement of Science, 1950, p. 109.
103. Ingbar, S. H., et al., *Proc. Second Clin. ACTH Conf.* **1**, 130 (1951).
104. Sprague, R. G., Mason, H. L., and Power, M. H., *Rec. Progr. Hormone Res.* **6**, 315 (1950).
105. Thorn, G. W., Jenkins, D., and Laidlaw, J. C., *Rec. Progr. Hormone Res.* **3**, 171 (1953).
106. Moehlig, R. C., and Steinbach, A. L., *J.A.M.A.* **154**, 42 (1954).
107. Perera, G. A., Ragan, C., and Werner, S. C., *Proc. Soc. Exper. Biol. & Med.* **77**, 326 (1951).
108. Ingle, D. J., and Baker, B. L., *Physiology and Therapeutic Effects of ACTH and Cortisone*. Springfield, Ill., Thomas, 1953.
109. Thorn, G. W., Laidlaw, J. C., and Goldfien, A., *CIBA Found. Coll. Endocrinol.* **3**, 343 (1955).
110. Levitt, M. F., and Bader, M. E., *Am. J. Med.* **11**, 715 (1951).
111. Laidlaw, J. C., Dingman, J. P., Arons, W. L., Finkenstaedt, J. T., and Thorn, G. W., *Ann. New York Acad. Sc.* **61**, 315 (1955).
112. Boland, E. W., *California Med.* **77**, 1 (1952).
113. Thorn, G. W., Engel, L. L., and Lewis, B. A., *Science* **94**, 348 (1941).
114. Hills, A. G., Pearsons, D. W., Rosenthal, O., and Webster, G. D., *J. Clin. Invest.* **34**, 940 (1955).
115. Conn, J. W., Fajans, S. S., Louis L. H., and Johnson, B., *Proc. Second Clin. ACTH Conf.* **1**, 221 (1951).
116. Homburger, F., Abels, J. C., and Young, N. F., *Am. J. Med.* **4**, 163 (1948).
117. Benda, L., Rissel, F., Scholda, E., and Scholda, G., *Acta endocrinol.* **15**, 236 (1954).
118. Pearson, et al., *J. Clin. Invest.* **30**, 665 (1951).
119. Bennett, L. L., Liddle, G. W., and Bentinck, R. C., *J. Clin. Endocrinol.* **13**, 392 (1953).
120. Dragsted, P. J., and Hjorth, N., *Scandinav. J. Clin. & Lab. Invest.* **5**, 188 (1953).
121. Ulrich, F., Reinhart, W. O., and Li, C. H., *Acta endocrinol.* **10**, 97 (1952).
122. Liddle, G. W., Bennett, L. L., and Forsham, P. H., *J. Clin. Invest.* **32**, 1197 (1953).
123. Knowlton, A. I., *Am. J. Med.* **15**, 771 (1953).
124. Dahl-Iversen, E., Engdahl, I., Nasner, E., Paaby, H., Sørensen, B., and Tobiassen, T., *Acta chir. scandinav.* **109**, 176 (1955).
125. Knowlton, A. I., *Bull. New York Acad. Med.* **29**, 441 (1953).
126. Soffer, L. J., Eisenberg, J., Innaccone, A., and Gabrilove, J. L., *CIBA Found. Coll. Endocrinol.* **3**, 487 (1955).

127. Mader, G. P., and Iseri, L., *Am. J. Med.* **19**, 976 (1955).
128. Conn, J. W., and Louis, L. H., *Ann. Int. Med.* **44**, 1 (1956).
129. Berger, E. Y., Quinn, G. P., and Homer, M. A., *Proc. Soc. Exp. Biol. Med.* **76**, 601 (1951).
130. Emerson, K., Kahn, S. S., and Jenkins, D., *Ann. New York Acad. Sc.* **57**, 280 (1953).
131. Conn, J. W., and Louis, L. H., *J. Clin. Endocrinol.* **10**, 12 (1950).
132. Locke, W., Talbot, N. B., Jones, H. S., and Worcester, J., *J. Clin. Invest.* **30**, 325 (1951).
133. Prader, A., Gautier, E., Gautier, R., Näf, D., Semer, J. M., and Rothschild, E., *CIBA Found. Coll. Endocrinol.* **8**, 382 (1955).
134. Grad, B., *J. Clin. Endocrinol.* **12**, 708 (1952).
135. Warming-Larsen, A., Hamburger, C., and Sprechler, M., *Acta endocrinol.* **11**, 400 (1952).
136. Steinberg, C., and Roodenburg, A. I., *Ann. Int. Med.* **44**, 316 (1956).
137. Pechet, M. M., *J. Clin. Invest.* **34**, 913 (1955).
138. Bunin, J. J., Black, R. L., Bollet, A. J., and Pechet, M. M., *Ann. New York Acad. Sc.* **61**, 358 (1955).
139. Liddle, G. W., Pechet, M. M., and Bartter, F. C., *Science* **120**, 496 (1954).
140. Goldfien, A., Beigelman, P., Laidlaw, J. C., and Thorn, G. W., *J. Clin. Invest.* **14**, 782 (1954).
141. Beck, J. C., *Ann. Int. Med.* **43**, 667 (1955).
142. Goldfien, A., Laidlaw, J. C., Haydar, A., Renold, A. E., and Thorn, G. W., *New England J. Med.* **252**, 415 (1955).
143. Liddle, G. W., and Richard, J. E., *Science* **123**, 324 (1956).
144. Gaunt, R., Loyd, Ch. W., and Charl, J. J., *Proc. Colston Res. Soc.* **8**, 233 (1956).
145. Li, M. C., Bergenstal, D. M., and Parrot, R. H., *J. Clin. Invest.* **35**, 721 (1956).
146. Duncan, L. E., Bartter, F. C., and Liddle, G. W., *Circulation* **12**, 697 (1955).
147. Bartter, F. C., Liddle, G. W., Duncan, L. E., and Delea, C., *J. Clin. Invest.* **35**, 688 (1956).
148. Muller, A. F., Riondel, A. M., Manning, E. L., and Mach, R. S., *J. Clin. Invest.* **35**, 725 (1956).
149. Venning, E. H., Singer, B., Caballeira, A., Dyrenfurth, I., Beck, J., and Giroud, C. P., *CIBA Found. Coll. Endocrinol.* **8**, 190 (1955).
150. Bartter, F. C., *Metabolism* **5**, 369 (1956).
151. Ward, L., and Hench, P. S., *Ann. New York Acad. Sc.* **61**, 620 (1955).
152. Wettstein, A., *Verhandlg. Schweiz. Naturforschenden Ges., Basel*, **136**, 22 (1956).

# The Properties, Estimation Methods, Hematologic Features, and Some Other More General Aspects of Different Abnormal Human Hemoglobins

*T. H. J. Huisman*

SINCE THE DESCRIPTION by Pauling and his associates (1) of a specific sickle cell hemoglobin and the introduction into medicine of the concept of the principle of "molecular diseases," rapid progress in the study of hemoglobin abnormalities has been made during the last 7 years. So it seems desirable to review the recent developments in this field. Several excellent general reviews appeared recently (2-7), dealing with various aspects of hemoglobin and its variants. This paper is therefore limited to a short review of some of the most important differences in the properties of the human hemoglobins, the technics for their identification and some interesting hematologic features. No extensive review of the clinical characteristics of the hereditary hemolytic syndromes is given; only a few general aspects will be discussed. Finally, some genetic features of the hemoglobin abnormalities and the incidence of the human hemoglobin types in different parts of the world will be given.

## TERMINOLOGY AND BIOCHEMISTRY OF HUMAN HEMOGLOBIN TYPES

At present 9 abnormal human hemoglobins next to the normal adult and fetal types have been described. They are listed in Table 1. With regard to the nomenclature of the different types, the recommendations of the committee of the hematology study section of the United States Public Health Service (8) are generally followed. The abnormal met-Hb, described by Hörlein and Weber (9) in 1948 and the possible abnormal hemoglobin discovered by Battle and Lewis (17) are not mentioned in the table.

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Table 1. DIFFERENT KINDS OF HUMAN HEMOGLOBIN DESCRIBED AT PRESENT TIME

<i>Nomenclature</i>	<i>First authors</i>	<i>Described in the following combinations</i>
Hb-A		
Hb-S (1)	L. Pauling c.s.	(1949) A+S, S+S, C+S, D+S, A+S+F.
Hb-C (10)	H. A. Itano + J. V. Neel	(1950) A+C, C+C, C+S, A+C+F.
Hb-D (11)	H. A. Itano	(1951) A+D, S+D.
Hb-E (12)	H. A. Itano c.s.	(1954) A+E, E+E, A+E+F.
Hb-F	E. Körber	(1866) Before, and a few months after birth, in pathological conditions: A+F, A+S+F, A+C+F, A+E+F.
Hb-G (13)	G. H. Edington + H. Lehmann	(1954) A+G, G+G.
Hb-H (14)	D. A. Rigas c.s.	(1955) A+H.
Hb-I (15)	E. B. Page c.s.	(1955) A+I.
Hb-J (16)	O. A. Thorup c.s.	(1955) A+J.
Hb-K (17a)	R. Cabannes c.s.	(1956) A+K.

As recommended by special committee of Hematology Study Section, States Public Health Service, January 1953.

A number of monographs and reviews has appeared in recent years dealing with the biochemistry of the several types of human hemoglobin (2, 4, 6, 18-20). Therefore I will confine myself to a summary of the most important differences in the biochemical characteristics of the several human hemoglobins. This comparison is given in Table 2. From these data it will be clear that numerous differences exist between fetal and adult hemoglobin, for instance in electrophoretic and chromatographic behavior, in ultraviolet spectroscopy, in the resistance to alkaline reagents, in solubility, in immunologic behavior. The results of the analyses of the amino acid composition (21), N-terminal residues (22-24), total half cystine content, and the number of free -SH groups (25) suggested that the structure of fetal hemoglobin differs markedly from that of the normal adult type. It may be that the fetal hemoglobin is built up by two different polypeptide chains, each with one valine residue in the N-terminal position and one tyrosine or one histidine residue in the C-terminal position (26), and connected to each other by one disulfide bridge. It is rather surprising that this Hb type which possesses so many distinctive biochemical properties is fully identical with the normal adult Hb in other characteristics such as molecular weight, heme groups, oxygen dissociation curve, and others.

The abnormal hemoglobins show a much lower number of characteristics that differentiate them from the normal adult pigment. The most general differences lie in the electrophoretic and chromatographic behavior, while some types of hemoglobin exhibit more specific differences, such as the low solubility of Hb-S in reduced state, the higher amount of lysine in Hb-C (27) and the instability of Hb-H. As far as studied almost identical results are obtained for

Table 2. COMPARATIVE BIOCHEMICAL CHARACTERISTICS OF THE HUMAN HEMOGLOBINS

Technic	Types									
	A	S	O	D	E	F	G	H	I	J
Electrophoretic mobility ( $\times 10^{-5}$ cm <sup>2</sup> /volt/sec.)	2.4	2.9	3.2	2.9	3.05	2.4	—	—	1.7	—
<i>pH</i> 6.5										
Isoelectric points COHb	6.87	7.09	7.30	7.09	7.20	6.98	—	5.60	Between A and H	Between A and I
Relative mobility on paper electrophoresis										
<i>pH</i> 8.6	6	4	2	4	3	5-6	5	>6	>6	>6
Chromatography on Amberlite 1RG (50)	1.00	0.75	0.33	0.75	0.90	1.33	0.75†	>1.4	1.15	1.00
Resistance to alkali denaturation	Low	Low	Low	Low	Low	High	Low	Low	Low	—
Tryptophane band (U.V.)	2910 °A	Like A	Like A	Like A	Like A	2898 °A	Like A	Like A	Like A	—
Solubility	COHb High	High	High	High	High	Higher	High	—	High	—
Reduced Hb	High	Very low	High	High	High	Higher	—	Low	—	—
Amino acid composition		Like A	Like A Lys++ Hist.+-	Like A	Like A	Threo.++Ser.++ Meth.+Isoleu.++ Trypt.+ Prol.-Val.- Tyr.-Hist.-	2 Valyl Like A	—	—	—
N-Terminal residues	5 Valyl	5 Valyl	5 Valyl		5 Valyl					
C-Terminal residues	1 Hist.	Like A	Like A							
(carboxypeptidase)	1 Tyr.									
Total cystine/2	8	8	8			6				
Free-SH groups	8	8	8			4				
Immunologic specificity	No	No	No			Yes				

the analyses of the amino acid composition (28, 29) the N-terminal (23, 24) and C-terminal (26) residues and the total half cystine and free -SH content (25). So in contrast with the fetal hemoglobin no clear picture can be obtained at the present time about possible differences in structure between the abnormal hemoglobins and the normal adult pigments.

### PHYSICOCHEMICAL METHODS FOR THE IDENTIFICATION OF DIFFERENT HUMAN HEMOGLOBINS

It will be clear that the physicochemical methods for identifying different hemoglobin types are based upon some different biochemical characteristics mentioned before. The most important and most generally useful methods are based upon differences in electrophoretic behavior. Also a good separation of some hemoglobins can be obtained in cation exchange chromatography. Furthermore some specific methods are available such as the determination of the solubility (especially for identifying the sickle cell hemoglobin). The fetal hemoglobin requires special interest, as this Hb type shows some highly specific characteristics, which may be useful for the estimation of this pigment. In this section we now will discuss these different methods.

#### THE MOVING BOUNDARY METHOD

Using the Tiselius apparatus and different buffer solutions it is possible to separate many abnormal hemoglobins from the normal adult type. Many authors (30-32) give exact descriptions of some systems useful in distinguishing different hemoglobin types. Good separations may be obtained with the following procedure:

For the preparation of a pure hemoglobin solution of approximately 10 Gm. Hb per 100 ml. from a sample of venous blood, the well-known method of washing the erythrocytes with saline and hemolyzing them with distilled water and toluene may be used. However it seems important to filtrate the hemoglobin solution finally over Celite 535 in order to remove all stroma proteins and some other impurities (21). The hemoglobin solution obtained is diluted with the buffer solution required to a final concentration of 1 to 2 Gm. per 100 ml. and dialyzed for 48 hours at 0° C. against the same buffer. The following buffer solutions are mostly used:

Veronal buffer, ionic strength 0.06, pH 8.8	} oxyHb
Phosphate buffers, ionic strength 0.1, pH 6.5; 6.75; 8.8	
Cacodylate buffer, ionic strength 0.1, pH 6.5	
	CO-Hb

When the electrophoresis was done in a standard Tiselius cell at 0-3° C., the potential gradient has to be about 6 to 8 volts per sq. cm.

From the data of Table 2 and Fig. 1, it will be clear that the separation of many abnormal hemoglobins is quite satisfactory. Moreover, with this method the specific mobility constants and the isoelectric points can be established.



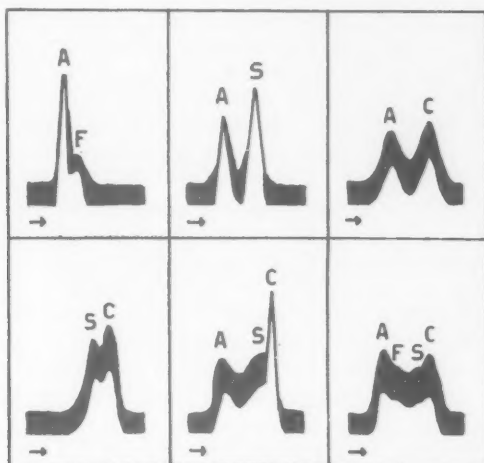


Fig. 1. The separation of different human hemoglobin types by moving-boundary electrophoresis.

However the abnormal hemoglobin D cannot be distinguished from the sickle cell pigment. Also with an artificial mixture (Hb-A, Hb-S, Hb-C, and Hb-F) only partial resolution of the different components is possible (see Fig. 1). A great advantage of the moving boundary technic is the possibility of getting quantitative data. However the relatively long time required for one analysis, the possibility of analyzing only one hemoglobin sample per day, and the expensiveness of the equipment are real restrictions of the method.

Although it seems possible to estimate most of the known abnormal hemoglobins with the procedure summarized here, it will be kept in mind that the study of the electric mobility of a hemoglobin sample over a wide range of pH values is necessary for recognizing the protein qualitatively. This is especially the case for the search after unknown abnormal hemoglobins in obscure hemolytic syndromes.

#### PAPER ELECTROPHORESIS

The zone electrophoresis on filter paper has been used by almost every worker for the study of the presence of an abnormal hemoglobin. A number of different apparatuses have been employed. Some investigators use a freely hanging nonhorizontal strip or sheet, others use supported horizontal paper. Probably most useful is a sheet of filter paper inserted between two siliconized glass plates (Fig. 2). The apparatus given there is about the same as described by many other investigators (e.g., [4]). Most satisfactory results were obtained using a sheet of Whatman 3 MM filter paper (45 cm. x 15 cm.) which has been

saturated with 0.06 ionic strength barbital buffer  $pH$  8.8. About 0.001 ml. of an approximately 10 Gm. per 100 ml. solution of monocarboxy hemoglobin is applied at the center of the paper (about 8 samples on one sheet). With a relatively high constant voltage (300–400 volt), a satisfactory resolution is obtained after 6 to 10 hours. Different dyeing procedures are available; for instance with bromphenol blue and with Amidoblack 10B.

The relative mobility on paper electrophoresis for the various hemoglobin types are given in Table 2, while Fig. 3 presents patterns of six abnormal hemoglobins (S, C, D, E, F, and one which belongs to the hemoglobins, moving faster than the adult pigment, Hb-J). It will be clear that many abnormal hemoglobins (C, E, S, or D and H, I or J) are separated easily from normal adult hemoglobin. Hb-F and Hb-G however migrate only a little more slowly than Hb-A, while Hb-D is indistinguishable from Hb-S and Hb-H from Hb-I by the procedure mentioned here. Although the best results are obtained with the veronal buffer  $pH$  8.8, sometimes the use of other buffers may be necessary. So it is possible to differentiate Hb-H from Hb-I by their paper electrophoretic patterns, using a phosphate buffer  $pH$  6.5, ionic strength 0.1 (33). In that case Hb-I shows about the same relative mobility as Hb-A, while Hb-H is easily distinguishable from the normal adult component.

A disadvantage of paper electrophoresis as compared with the boundary method is its inability to provide absolute mobility data. Moreover, accurate quantitative data are not always obtainable. Even as for serum proteins this problem has been the subject of much debate. The two general methods are used: direct transmission scanning of the dry undyed strips at 5200 Å (34) or dyeing with bromphenol blue and eluting the dye (35). They seem to be useful for semiquantitative purposes, although "tailing" of the compounds on the paper may also prevent accurate quantitative data.

Because of the simplicity of the procedure, paper electrophoresis is ideally suited for mass surveys, since a nearly unlimited number of samples can be investigated simultaneously. Especially for anthropologic and genetic studies, the method is highly valuable. Moreover, as the equipment is not expensive and no trained personnel are required, the technic is available to every average hospital laboratory.

A modification of the electrophoretic method will be briefly mentioned.

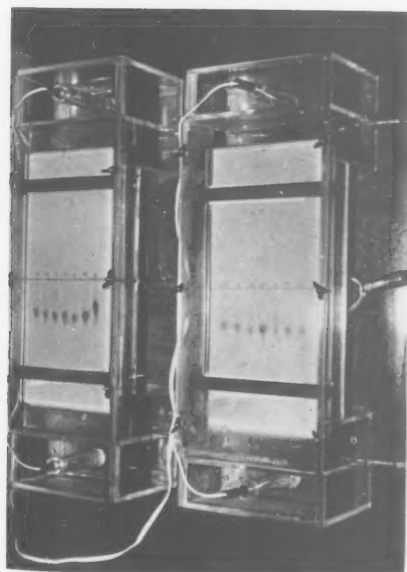
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**Fig. 2.** Paper electrophoresis apparatus.

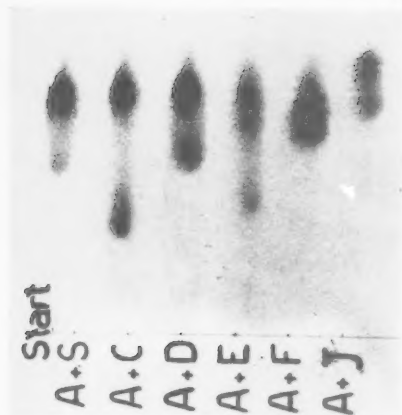
**Fig. 3.** The separation of different hemoglobin types by paper electrophoresis. Whatman 3MM, barbital buffer  $pH$  8.8, ionic strength 0.06.

**Fig. 5.** The separation of five different human hemoglobins on flat Lucite columns filled with Amberlite IRC 50 (XE 64).

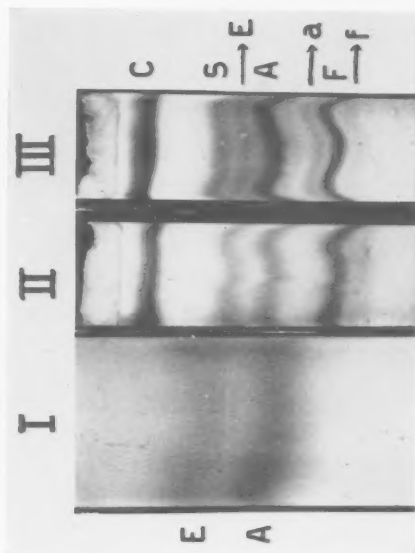
**Fig. 6.** Application of the chromatographic method in blood samples of some patients with hemoglobin abnormalities. I, Cooley trait. II, Cooley trait after addition of Hb-F. III, Cooley's anemia. IV, Sickle-cell trait. V, Heterozygous Hb-C disease.



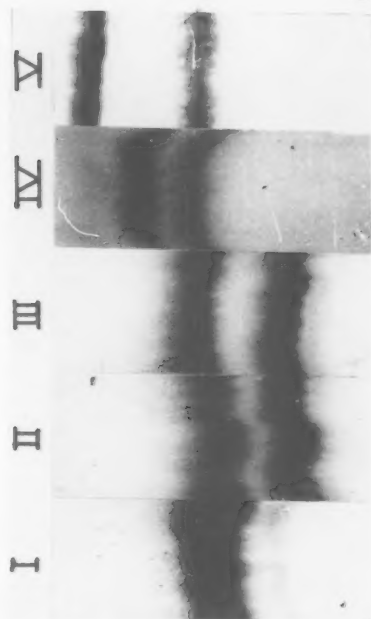
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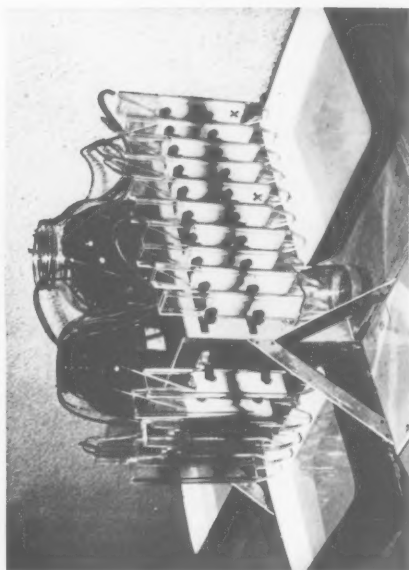
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5



6



7



12



13

This technic, which was recently described by Tuttle (88), is based upon the formation of "isoelectric line spectra," when different abnormal hemoglobin types are interposed between two buffer systems, one with a relatively high density and a pH below the isoelectric points of the components and the other with a pH above these points. Upon the application of current, the hemoglobins condense in horizontal lines according to their isoelectric points. The separation of the lines depends upon the differences in these points, but also upon the slopes of the pH and density gradients between the two buffer solutions. The method seems valuable for the identification of different hemoglobin types. Moreover, the apparatus described by the author is very simple and not expensive, while the short time for separation (about 30 minutes) is a great advantage of the procedure.

CHROMATOGRAPHIC SEPARATION USING THE CATION EXCHANGE RESIN,  
AMBERLITE IRC 50 (XE 64)

Thanks to the fundamental work of Boardman and Partridge (36) on the chromatography of hemoglobins and related proteins on cation exchange resins, a procedure was developed for the separation of some different hemoglobins in this way (37, 38). It was found that under certain experimental conditions the adsorption rates of some hemoglobin types on the cation exchanger Amberlite IRC 50 (XE 64) were different. Due to this fact it was possible to completely separate four different hemoglobins (37, 38) on a small column (13 cm. x 0.9 cm.), using citrate buffers of increasing sodium ion concentration (Fig. 4, p. 380). As this technic, however, was not suitable for routine analyses, another simplified method was worked out. This procedure has been described elsewhere in detail (38). It may be summarized as follows: Flat hollow lucite columns (20 cm. x 3 cm. x 0.5 cm. inside) were filled to 15 cm. with the resin, which was pretreated with 4N NaOH, distilled water, 4N HCl, and distilled water again in the way described elsewhere (39). The column was pretreated with a sodium citrate buffer pH 6.0, sodium ion concentration 0.15. About 10 mg. of COHb (in 4 ml.) was added to the surface of the column and the chromatogram was developed in about 10 hours by siphoning the same sodium citrate buffer through the column with a flow rate of about 20 ml. per hour.

Figure 5 (p. 377) represents some of the directly visualized results of the

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Fig. 7. The use of the chromatographic procedure on a large scale. All hemoglobin samples investigated contained Hb-A, except two, obtained from patients suffering from a sickle-cell trait (marked with X).

Fig. 12. Family with two members (mother and one child) possessing the abnormal hemoglobin J.

Fig. 13. Family with the Hb-E trait (mother and two daughters), Cooley trait (father and the youngest son), and the thalassemia Hb-E disease (eldest son and youngest daughter, the latter not shown).

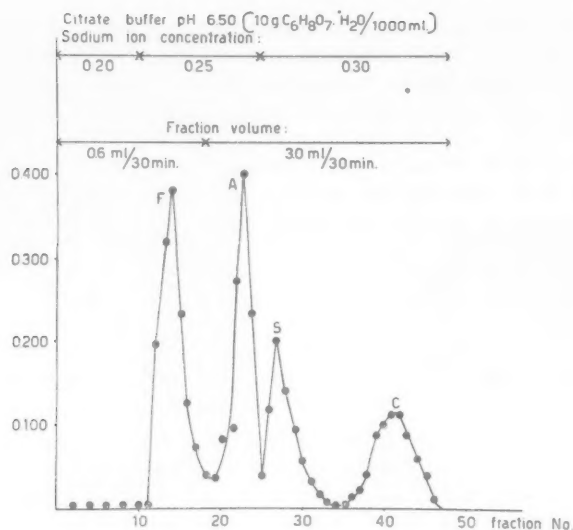


Fig. 4. Elution curve of four different human hemoglobins on a column of Amberlite IRC 50 (XE 64).

separation of four different hemoglobin types (F, A, S, and C) and also of Hb-E, whose mobility is only slightly less than that of Hb-A (40). The different bands may be identified by their different rates of displacement, given in Table 2, or by adding known hemoglobins. Next to the five bands some small zones appeared with flow rates somewhat higher than the mean bands. It may be that these zones are due to the inhomogeneity of hemoglobin in cation exchange chromatography, some indications of which have appeared recently in the literature (41). In other experiments it was found that Hb-D and perhaps Hb-G behave like Hb-S. Hemoglobin J has the same mobility as Hb-A, while Hb-I moves faster than the adult component.

In Fig. 6 (p. 377) the results are given of the analyses of hemoglobin in some hemolytic disorders associated with abnormal hemoglobins. From these pictures it will be clear that the chromatographic procedure can be successfully applied to the estimation of fetal hemoglobin, as this hemoglobin is completely separated from the normal adult type. In this respect this technique is preferable to the electrophoretic procedure.

A semiquantitative estimation of the percentages of the different hemoglobin types present in a mixture may be carried out by measuring a diapositive of the cuvet in the same way as the scanning method in paper electrophoretic analyses. Some results for different artificial mixtures of Hb-A and Hb-F

are given in Table 3. The data in this table show that the range of error in the determination is 5 to 10 per cent.

The chromatographic procedure is also successful for large-scale analyses (Fig. 7, p. 378). Thirty to 40 columns may be prepared in about 2 hours by one person, while development is carried out overnight. The method is therefore suitable for average hospital laboratories.

#### SOLUBILITY DETERMINATIONS

As the solubility of some hemoglobins in salt solutions differs from that of the normal adult hemoglobin, salting-out experiments are valuable for identification. This is especially the case for Hb-D, because the sickle cell hemoglobin differs only in solubility from this hemoglobin in the reduced state. The procedure described by Derrien and co-workers (42, 43) using phosphate buffers pH 6.5 in various concentrations may be an exact method in establishing the solubility of the different forms (monocarboxy-, reduced) of a hemoglobin type (Fig. 8, p. 382). However, due to technical complication this method does not lend itself for routine analysis. Moreover, as solubility tests are only necessary in differentiating Hb-S from Hb-D, the simpler method of Itano (44) is preferred. The principle is as follows: in an exact volume of 10 ml. 50 mg. hemoglobin are incubated at 25° C. with a phosphate solution (final concentrations 2.24 molar or 2.58 molar). When Hb-S is present in the hemoglobin sample, a precipitate is formed. The amount of hemoglobin remaining in solution is estimated spectrophotometrically at 5400 Å. It was found that in the sickle cell trait, for instance, the solubility in a 2.24M phosphate solution ranges from 1.25 to 2.15 Gm. per liter and in sickle cell anemia from 0.10 to 0.95 Gm. per liter, while specimens in which Hb-S is absent dissolved completely (5 Gm. per liter). In this way the differentiation between Hb-S and Hb-D can easily be carried out.

Table 3. THE ESTIMATION OF FETAL AND ADULT HEMOGLOBIN IN MIXTURES OF COHb-A AND COHb-F ESTIMATED BY THE CHROMATOGRAPHIC METHOD AT 0°

Sample	Calculated		Cuvet Method	
	COHb-F	COHb-A	COHb-F (Per cent)	COHb-A (Per cent)
1	83.5 <sup>a</sup>	16.5	91.5; 91.5 <sup>b</sup>	8.5; 8.5 <sup>b</sup>
2	72.5	27.5	79 ; 77.5	21 ; 22.5
3	59	41	61 ; 61	39 ; 39
4	42.5	57.5	49.5; 47.5	50.5; 52.5
5	24	76	22 ; 19.5	78 ; 80.5
6	12	88	12.5; 12.5	87.5; 87.5

<sup>a</sup>Estimated by the alkali denaturation method of Brinkman and Jonxis (47); the percentages of Hb-F in the other five samples were obtained by dilution with normal adult hemoglobin.

<sup>b</sup>Duplicate estimations.

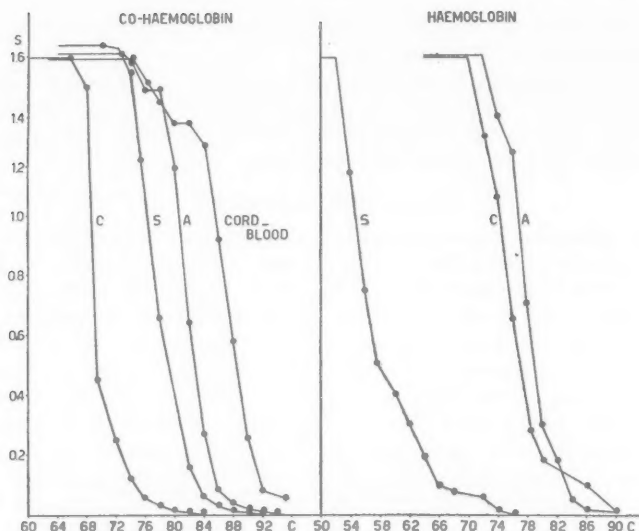


Fig. 8. Salting-out curves of various hemoglobin types.

#### ESTIMATION OF FETAL HEMOGLOBIN

With the four methods mentioned above, it is possible to estimate definitely the presence of most of the known abnormal hemoglobins. *The estimation of fetal hemoglobin* requires special attention, as this hemoglobin shows many specific properties upon which different methods are based. The most important one is the *high resistance against alkali*, already described, in 1866, by Körber. Different technics based upon this characteristic property are used at the present time. They are to be divided into two groups:

1. The procedures at which the alkali denaturation product is salted-out and removed by filtration comprise the first group. With the method of Singer *et al.* (45), for instance, the hemoglobin, which is denatured by 0.1N NaOH in the first minute of the reaction and which consists mainly of the adult component, is precipitated by adding certain amounts of a half-saturated ammonium sulfate solution. The remaining hemoglobin, mainly fetal hemoglobin, is measured spectrophotometrically. Recently Künzer (46) described a new method based on alkali denaturation starting from cyanhemoglobin, also using precipitation. The method of Singer *et al.* has especially found wide application.

2. The methods of the second group are based on the same principle. However, with these technics the percentage of fetal hemoglobin present is established by spectrophotometrically measuring the slowly disappearing quantity of the alkali-resistant hemoglobin. As the alkali denaturation of Hb-F behaves like a monomolecular reaction, the logarithms of the percentages of the unde-



natured hemoglobin at different moments plotted against time will form a straight line. The percentage of Hb-F present in the sample can easily be calculated by extrapolation to zero time. In 1935, Brinkman and Jonxis (47) had already described this method, while Beaven, Hoch, and Holiday (48) used similar procedures. Two wavelengths are used in this respect: 6500 Å and 5760 Å (49). As pointed out by Jonxis and Visser (50) the spectrophotometric method at 5760 Å seems to be more accurate especially for the determination of low percentages of fetal hemoglobin (Fig. 9).

In comparing the two alkaline denaturation procedures it was found (51) that with the method of Singer *et al.* high percentages of Hb-F are too low and low percentages too high (Fig. 10). Moreover, the presence of small percentages of carboxy hemoglobin (drivers, heavy smokers) will interfere with the determination of Hb-F, as the monocarboxy compound shows a much higher resistance against alkali than the oxyhemoglobin (49, 50). This influence is especially important, when using the direct spectrophotometric methods. So I believe that both methods are not well suitable for the estimation of low percentages (below 5 per cent) of Hb-F, while also a relatively large deviation for samples with higher amounts of fetal hemoglobin will be taken into account. Perhaps the spectrophotometric determination at 5760 Å may be the most accurate method at present time.

Next to the alkali denaturation procedures other methods are available. It is, however, questionable whether these procedures are more accurate. In the preceding section I mentioned already the *moving boundary electrophoretic method* and the *chromatographic procedure with the cation exchanger Amberlite 1RC 50 (XE 64)*. Other methods based on other specific properties of Hb-F and sometimes used are: the *spectrophotometric method* based on the

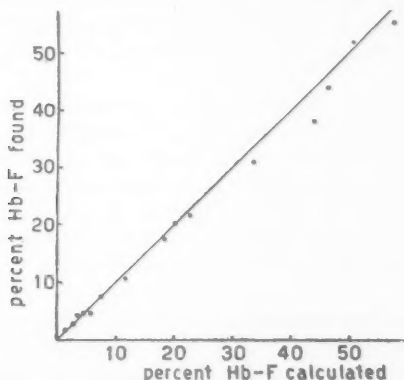


Fig. 9. Fetal hemoglobin concentrations estimated by the optical alkali denaturation method at 5760 Å.

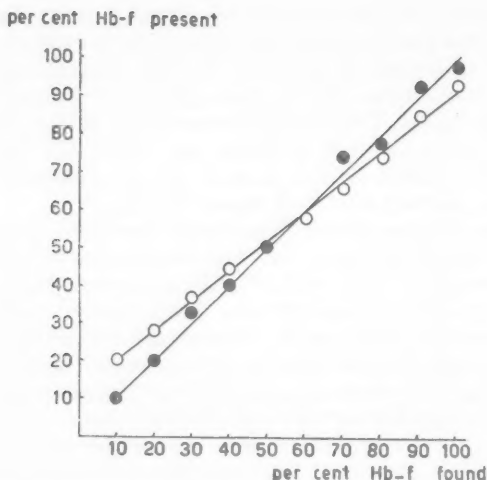


Fig. 10. Comparison between the results of the estimation of different Hb-F percentages obtained with the precipitation method (O O O) and the optical method at 6500 Å (● ● ●).

difference in the tryptophane band in the ultraviolet spectral absorption (52). The accuracy of this method is limited to 5 to 10 per cent of Hb-F (Fig. 11), even with the use of refined methods such as the moving-plate ultraviolet spectrophotometric method (53).

It is somewhat difficult to interpret the results of *salting-out experiments* (43) especially of hemoglobin mixtures with relatively low amounts of Hb-F. It may be that low percentages of Hb-F are to be estimated in this way: however the possible inhomogeneity of hemoglobin in these experiments confuses the final results.

Finally, with the *immunologic procedure* of Chernoff (54), a method is obtained in estimating small amounts of fetal hemoglobin in blood samples.

The relatively large *differences in the amino acid composition* between Hb-A (and the other abnormal hemoglobins) and the fetal pigment (21) also offer a method for identifying the fetal hemoglobin. Especially the great difference in isoleucine content may be useful in this respect. With this procedure the presence of small amounts of fetal hemoglobin, in normal adults, already mentioned by other investigators (43, 45, 54) was more definitely confirmed (55). Also more evidence was obtained that the alkali-resistant hemoglobin in Cooley's anemia was identical with the fetal component (56).

Although many possibilities exist for the estimation of Hb-F, the procedures available for an average hospital laboratory are restricted to one of the alkali-denaturation methods and the chromatographic procedure with the cation

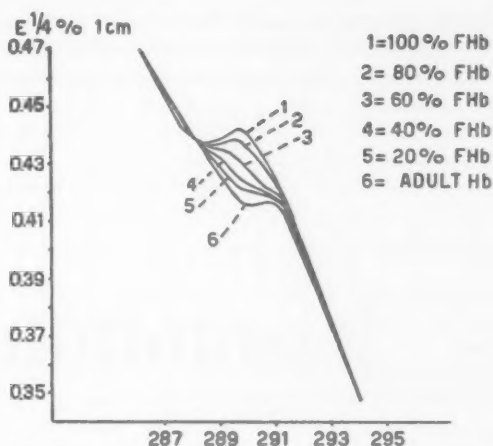


Fig. 11. The estimation of different percentages of fetal hemoglobin by the ultraviolet spectrophotometric method based on the difference in the tryptophane band between Hb-A and Hb-F.

exchanger Amberlite IRC 50 (XE 64). When the technical equipment is present, the moving-boundary method is also useful in this respect.

### CLINICAL ASPECTS

Many unknown hemolytic disorders may be explained at the present time by the presence of abnormal hemoglobins or by combinations of some hemoglobin types. Next to these combinations the thalassemia syndrome and the syndromes consisting of an abnormal hemoglobin combined with other hereditary defects such as thalassemia have to be mentioned. Without going into a detailed description of the clinical data—they are available in the literature ([4-6] among others)—I will briefly review a few distinctive hematologic and clinical characteristics of these disorders. They are summarized (Table 4) here only in view of the possibility of detecting an abnormal hemoglobin by these features. The genetic representation proposed by Allison (75) and discussed later, is followed. From the data given in the table it will be clear that in general they are insufficient for definite recognition of an abnormal hemoglobin. Only the sickle cell phenomenon is a specific characteristic. Relatively high percentages of target cells points to the presence of the abnormal hemoglobins C or E. Severe anemia was found in the homozygous sickle cell anemia, in Cooley's anemia, and in the thalassemia Hb-S and thalassemia Hb-E disease. Most of these anemias are microcytic and hypochromic with a large reticulocytosis. In most disorders associated with an abnormal hemoglobin type the osmotic fragility is increased.

**Table 4.** SOME CLINICAL AND HEMATOLOGIC DATA FOR CERTAIN INHERITED ABNORMALITIES OF THE BLOOD ASSOCIATED WITH ABNORMAL HEMOGLOBINS

	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>	Hb <sup>a</sup> /Hb <sup>b</sup> /Th <sup>c</sup>
Total Hb content																		
Kind of Hb present	A	A	S	S	S	A	C	A	A	E	A	G	A	A	A	A	A	A
Target cells	-	+	+	++	+	+	++	-	+	++	-	+	+	+	+	+	+	++
Sickle cells	-	+	++	+	+	-	-	-	-	-	-	-	-	-	-	-	++	-
Reticulocytosis	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+
Resistance to hypotonic saline	0	±	+	+	+	+	±	+	+	0	+	+	0	+	±	+	+	+
Splenomegaly	-	+	+	+	-	±	+	-	±	+	+	-	+	-	+	±	+	+

It is common knowledge that the clinical and hematologic abnormalities are less severe in the heterozygous hemoglobin-trait syndromes. It is remarkable that the clinical picture in patients with some rare abnormal hemoglobin traits (Hb-G trait, Hb-J trait) is practically normal. We recently investigated many families of Indonesian extraction and found in one family, in which the mother came from Java and the father from Ambon, an abnormal hemoglobin, which migrates faster in paper electrophoresis (barbital buffer pH 8.8, ionic strength 0.06) than the normal adult component. The family is shown in Fig. 12 (p. 378) and the electrophoretic pattern in Fig. 3 (p. 377). Comparison of this hemoglobin with Hb-H, Hb-I, and Hb-J<sup>1</sup> revealed that the abnormal hemo-

<sup>1</sup>The author is very much indebted to Drs. Rigas, Portland, Rucknagel, Durham, and Itano, Bethesda, Md., for the comparative studies of this Hb sample with Hb-H, Hb-I, and Hb-J.

globin was most likely identical with Hb-J. It is remarkable that no clinical symptoms and hematologic deviations were found (Table 5). The question arises if the presence of an abnormal hemoglobin in individuals, who are fully normal in many respects, may bring on any complications. In the children of a marriage of such a patient with a partner who possesses an abnormal hemoglobin (Hb-S, thalassemia), which in the homozygous state cause a severe anemia, it is possible that difficulties occur. When a child inherits a single gene for the one abnormal hemoglobin and a single gene for the second, it may happen that the presence of the first factor causes about the same clinical manifestations as found in the homozygous state of this hemoglobin abnormality. In this respect, the combinations of the thalassemia gene with Hb-S, Hb-C, or Hb-E (58-61, 63) are known. Especially patients with the combination of the thalassemia gene and the Hb-E gene are of interest, since the heterozygous Hb-E trait is not associated with specific clinical hematologic or physical abnormalities. The children with the thalassemia Hb-E disease, however, show clinical symptoms similar to those of thalassemia major in almost every respect. This will be clear for instance from the data obtained of a family we studied sometime ago (29). The family (Fig. 13, p. 378) was from Indonesia: the father (British-Javanese extraction) was suffering from a Cooley trait and the mother (Indonesian-Chinese extraction) from the heterozygous Hb-E disease. Of the 5 children (Fig. 14) 2 had an Hb-E trait, 1 a Cooley trait, while 2 showed the clinical picture of a moderate-severe Cooley's anemia (Table 6). Since the blood of these 2 patients contained up to 40 per cent Hb-E, 30 to 40 per cent Hb-F, and Hb-A, it was clear that they were suffering from thalassemia-Hb-E disease. The possibility arises that the same occurs in other hemoglobin combinations not found at the present time, and this may explain still unknown hemolytic disorders. For these reasons it seems

**Table 5.** SOME HEMATOLOGIC DATA OF A FAMILY WITH TWO MEMBERS POSSESSING THE ABNORMAL HEMOGLOBIN J

	Father K.	Mother K.	D.K.	J.K.	A.K.	M.K.
Age	33	31	6	4	3	1
Hb (Gm./100 ml.)	17.2	13.8	12.1	11.4	12.7	11.6
RBC (millions/cu. mm.)	6.5	5.2	4.5	4.0	5.1	4.7
Retic. %	1.1	1.1	1.7	2.0	1.9	1.6
WBC per cu. mm.	6700	11300	6400	8100	5500	10800
Osmotic fragility (% saline)	0.48-0.40	0.47-0.39	0.46-0.37	0.48-0.40	0.46-0.36	0.47-0.34
Target cells	0	0	0	0	0	0
Indirect serum bilirubin (mg./100 ml.)	0.9	1.1	0.8	0.4	0.5	0.7
Splenomegaly	Absent	Absent	Absent	Absent	Absent	Absent
Type of Hb	A	A+J	A	A+J	A	A

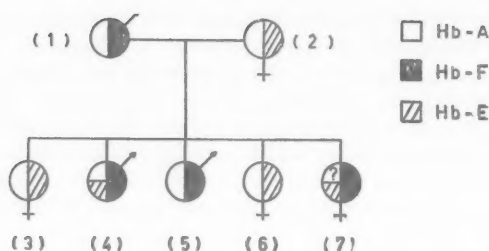


Fig. 14. Genetic representation of the family with the Hb-E trait, Cooley trait, and thalassemia Hb-E disease.

Table 6. SOME HEMATOLOGIC DATA OF A FAMILY WITH TWO MEMBERS WITH THE THALASSEMIA-Hb-E DISEASE

	Father	Mother	W. V.W.	Fr.W.	F.W.	H.W.	M.W.
Age	32	29	8	6	4	3	1
Hb (Gm./100 ml.)	14.1	12.8	11.6	5.7	10.9	9.6	8.4
RBC (milions/cu. mm.)	—	4.7	4.4	2.8	4.8	5.1	4.2
Retie. %	1.2	1.1	1.7	7.0	1.6	2.0	9.8
Osmotic fragility (% saline)	—	0.44-0.34	0.44-0.28	0.56-0	0.44-0.30	0.42-0.20	0.14
Target cells	±	0	0	+	0	±	+
Indirect serum bili- rubin (mg./100 ml.)	—	0.4	0.4	—	0.5	0.6	1.2
Splenomegaly	Absent	Absent	Absent	Removed	Absent	Absent	Present
Type of Hb	A+F	A+E	A+E	A+F+E	A+E	A+F	A+F+E

important to investigate large numbers of apparently normal individuals of different races using different methods.

### GENETIC PATTERNS

The most satisfactory explanation of the basic genetic features of the hereditary hemolytic disorders associated with abnormal hemoglobins is still the theory of Neel (64) that the determinants for pathologic hemoglobin types are alleles of the genes for normal adult hemoglobin. There is much evidence however that the thalassemia gene is not an allelomorph of the other genes affecting hemoglobin formations (57). Also the fact that the chemical structure of the fetal hemoglobin present in the blood of patients with Cooley's anemia is quite different from that of the normal adult component points into that direction. So it seems that the fetal hemoglobin production is controlled by genes which are not alleles for Hb-A, but are located at other loci of the chromosomes.

Even as for the nomenclature of the different hemoglobin types there is the need for a uniform system of designating the genetic types of the diseases associated with abnormal hemoglobins. Perhaps a good notation for the different

genes may be the symbols as suggested recently by Allison (57). In Fig. 15 some examples are given. In this notation the nonallelic pattern of the thalassemia gene is taken into account.

### INCIDENCE

Since the discovery of the sickle cell hemoglobin by Pauling and associates (1), much progress had been made in discovering the incidence of different hemoglobin types in the world. The chart given in Fig. 16 gives a review of our present knowledge.

Sickle cell hemoglobin is found in the Negro race all over the world; up to 45 per cent has been detected in some African tribes (65), although in other tribes it is practically absent (66). Extensive studies (for instance, Lehmann 67) suggest that the sickle cell Hb may have arisen as a mutation in the inhabitants of Southern Arabia or in the Vedddoid inhabitants of Southern India. From these parts it has been transported to Africa and other parts of the world.

The second Negro hemoglobin (Hb-C) is found in relatively high percentages (about 12 per cent) in the Gold Coast (68); in the United States it is present in about 2 to 3 per cent of the Negro population (69). The incidence of this hemoglobin in the northern part of Africa (70) and Curaçao (27) is also reported. It is completely absent in the Negro tribes of East Africa (71).

Only a few cases of Hb-D have been reported; in the United States (11),

NORMAL ADULT	$Hb^A Hb^A / Th^N Th^N$
HETEROZYGOUS STATE OF AN ABNORMAL Hb (f.i. Hb-S)	$Hb^A Hb^S / Th^N Th^N$
HOMOZYGOUS STATE OF AN ABNORMAL Hb (f.i. Hb-S)	$Hb^S Hb^S / Th^N Th^N$
THALASSEMIA TRAIT	$Hb^A Hb^A / Th^N Th^F$
THALASSEMIA MAJOR	$Hb^A Hb^A / Th^F Th^F$
THALASSEMIA Hb-X DISEASE	$Hb^A Hb^X / Th^N Th^F$
COOLEY Hb-X DISEASE (?)	$Hb^A Hb^X / Th^F Th^F$
(X = GENE FOR Hb-S, Hb-C or Hb-E)	

Fig. 15. Notation system for hemoglobin types and genes controlling their synthesis, according to Allison, A. C., *Science* **121**, 640 (1955).

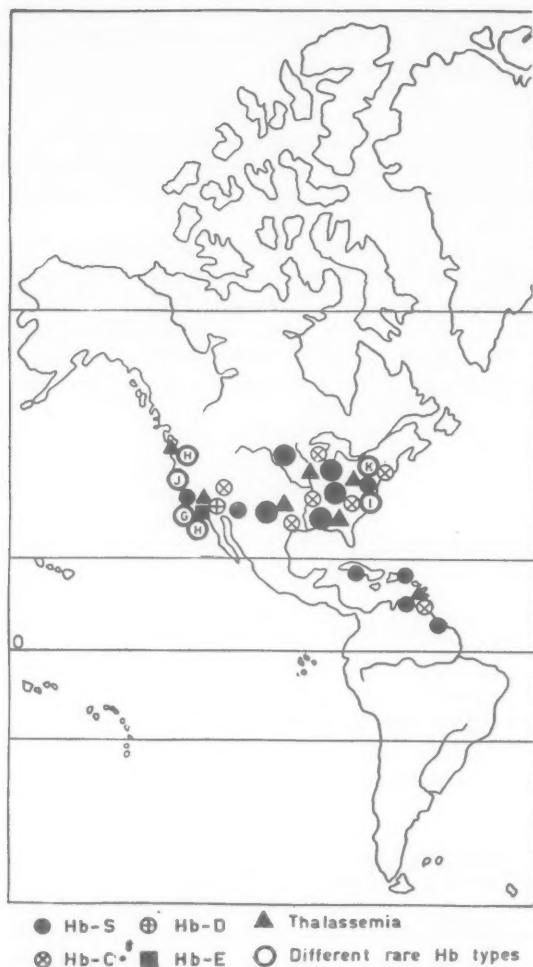


Fig. 16. Incidence of the different hemoglobin types in the western hemisphere (May, 1956).

England, (72, 73), Northern India (74), and Algeria (75). Recently Bird and Lehmann (89) found an incidence of about 1 per cent of this hemoglobin in Punjabis and in Gujeratis.

Hemoglobin E is found in different parts of Southeast Asia, e.g., Thailand



(12, 60, 63), Burma, (76), India, Ceylon (77), and Indonesia (61), while it was also established in a few families in Holland, who had immigrated from Indonesia (29).

Thalassemia is a common disease in different Mediterranean countries such as Italy (Ferrara, Sicily) and Greece. It is also frequently found in other parts of the world; for instance, India (78), Thailand (79), Indonesia (80). It has also been seen in China (81), Burma (82), in the northern parts of Persia (83), Iran (84) in Jews of Kurdistan (85) and in Negroes in the United States (86) and some parts of South America and Africa.

Beside the family living on the Gold Coast (13), only 1 case with the abnormal hemoglobin G has been described (87). The other hemoglobin types: Hb-H, (14), Hb-I (15), Hb-J (16), and Hb-K (17) have each reported only 1 case. Additional reports of other observations did not appear at the present time, but it seems likely that in other parts of the world these abnormal hemoglobins will be present. It will be kept in mind that many anthropologic studies are still in progress, while large parts of the world have not been sufficiently investigated.

Since the discovery of the abnormal Hb-S, striking progress has been made in the study of the defects due to the presence of an abnormal hemoglobin. A series of abnormal human hemoglobin types is now firmly recognized. Information on their biochemical, genetic, anthropologic, and clinical features has been obtained. Many methods useful in the characterization of these hemoglobins have been developed; most of them can be easily applied in every average hospital laboratory. However, it will be clear that further studies in this field may provide a deeper understanding of the significance of the hemoglobin types in medicine and in biochemistry and may give the possible explanation of other diseases which are not yet understood.

## REFERENCES

1. Pauling, L., Itano, A. H., Singer, S. J., and Wells, I. C., *Science* **110**, 543 (1949).
2. White, J. C., and Beaven, G. H., *J. Clin. Pathol.* **7**, 175 (1954).
3. Pauling, L., *Harvey Lectures* **49**, 216 (1954).
4. Chernoff, A. I., *New England J. Med.* **253**, 322 (1955).
5. Singer, K., *Am. J. Med.* **18**, 633 (1955).
6. Betke, K., *Klin. Wochenschrift* **34**, 113 (1956).
7. Itano, H. A., *Arch. Int. Med.* **96**, 287 (1955).
8. *Science* **118**, 116 (1953).
9. Hörlein, H., and Weber, G., *Deutsche Med. Wochenschr.* **73**, 476 (1948).
10. Itano, H. A., and Neel, J. V., *Proc. Nat. Acad. Sci. U. S.* **36**, 613 (1950).
11. Itano, H. A., *Proc. Nat. Acad. Sci. U. S.* **37**, 775 (1951).
12. Itano, H. A., Berggren, W. R., and Sturgeon, P., *J. Am. Chem. Soc.* **76**, 2278 (1954).
13. Edington, G. M., and Lehmann, H., *Lancet* **11**, 173 (1954).
14. Rigas, D. A., Koler, R. D., and Osgood, E. E., *Science* **121**, 372 (1955).
15. Page, E. B., Rucknagel, D. L., and Jensen, W. N., *Clin. Res. Proc.* **3**, 67 (1955).

16. Thorup, O. A., Itano, H. A., and Wheby, M. S., in Itano, H. A., *Arch. Int. Med.* **96**, 287 (1955).
- 16a. Thorup, O. A., Itano, H. A., Wheby, M. S., and Leavell, B. S., *Science* **123**, 889 (1956).
17. Battle, J. D., and Lewis, L., *J. Lab. Clin. Med.* **44**, 765 (1954).
- 17a. Cabannes, R., *C. S. Proceedings Vth Intern. Congress of the Intern. Soc. of Hematology*, Boston, 1956.
18. Lemberg, R., and Legge, J. W., *Hematin Compounds and Bile Pigments*, New York and London, Interscience, 1949.
19. Drabkin, D. L., *Physiol. Rev.* **31**, 345 (1951).
20. Roughton, F. J. W., and Kendrew, J. C., (Eds.) *Barcroft Memorial Volume Haemoglobin*. London, Butterworth, 1949.
21. van der Schaaf, P. C., and Huisman, T. H. J., *Biochim. Biophys. Acta* **17**, 81 (1955).
22. Porter, R. R., and Sanger, F., *Biochem. J.* **42**, 287 (1948).
23. Masry, M. S., and Singer, K., *Arch. Biochem. Biophys.* **58**, 588 (1955).
24. Huisman, T. H. J., and Drinkwaard, J., *Biochim. Biophys. Acta* **18**, 588 (1955).
25. Hommes, H., Santema-Drinkwaard, J., and Huisman, T. H. J., *Biochim. Biophys. Acta* **20**, 564 (1956).
26. Huisman, T. H. J., and Dozy, A., *Biochim. Biophys. Acta* **20**, 400 (1956).
27. Huisman, T. H. J., van der Schaaf, P. C., and van der Sar, A., *Blood* **10**, 1079 (1955).
28. Huisman, T. H. J., Jonxis, J. H. P., and van der Schaaf, P. C., *Nature* **175**, 902 (1955).
29. Jonxis, J. H. P., Huisman, T. H. J., van der Schaaf, P. C., and Prins, H. K., *Nature* **177**, 627 (1956).
30. Zinsser, H. H., *Arch. Biochem. and Biophys.* **38**, 195 (1952).
31. Beaven, G. H., Hoch, H., and Holiday, E. R., *Biochem. J.* **49**, 375 (1951).
32. Neel, J. V., Itano, H. A., and Lawrence, J. S., *Blood* **8**, 434 (1953).
33. Rucknagel, D. I., Page, E. B., and Jensen, W. N., *Blood* **10**, 999 (1955).
34. Motulsky, A. G., Paul, M. H., and Durrum, E. L., *Blood* **9**, 897 (1954).
35. Ranney, H. M., Larson, D. L., and McCormack, G. M., Jr., *J. Clin. Invest.* **32**, 1277 (1953).
36. Boardman, N. K., and Partridge, S. M., *Biochem. J.* **59**, 543 (1955).
37. Prins, H. K., and Huisman, T. H. J., *Nature* **175** 903 (1955).
38. Huisman, T. H. J., and Prins, H. K., *J. Lab. Clin. Med.* **46**, 255 (1955).
39. Hirs, C. H. W., Moore, S., and Stein, W. H., *J. Biol. Chem.* **200**, 493 (1953).
40. Prins, H. K., and Huisman, T. H. J., *Nature* **177**, 840 (1956).
41. Morrison, M., and Cook, J., *Science* **120**, 920 (1955).
42. Derrien, Y., *Bioch. Biophys. Acta* **8**, 631 (1952).
43. Roche, J., Derrien, Y., Reyman, J., Laurent, G., and Roques, M., *Bull. Soc. Chim. Biol.* **36**, 51 (1954).
44. Itano, H. A., *Arch. of Biochem. and Biophys.* **47**, 148 (1953).
45. Singer, K., Chernoff, A. I., and Singer, L., *Blood* **6**, 413 (1951).
46. Künzer, W., *Z. Kinderheilk.* **76**, 56 (1955).
47. Brinkman, R., and Jonxis, J. H. P., *J. Physiol.* **85**, 117 (1935).
48. Beaven, G. H., Hoch, H., and Holiday, E. R., *Biochem. J.* **49**, 374 (1951).
49. Betke, K., *Der Menschliche rote Blutfarbstoff bei Fetus und reifem Organismus*. Berlin, Springer Verlag, 1954.
50. Jonxis, J. H. P., and Visser, H. K. A., *Am. J. Dis. Child.* **92**, 588 (1956).
51. Huisman, T. H. J., and Jonxis, J. H. P., *Blood* **ii**, 1009 (1956).
52. Jope, E. M., in *Haemoglobin*, London, Butterworth, 1949, pg. 205.
53. Holiday, E. R., in *Analytical Absorption Spectroscopy* Mellon, M. G., (Ed.) New York, Wiley, pg. 268, Chapman and Hall, London.
54. Chernoff, A. I., *Blood* **8**, 399 (1953).

55. Huisman, T. H. J., Jouxis, J. H. P., Dozy, A., *Biochim. Biophys. Acta* **13**, 576 (1955).
56. Huisman, T. H. J., Prins, H. K., and van der Schaaf, P. C., *Experientia* **12**, 107 (1956).
57. Allison, A. C., *Science* **121**, 640 (1955).
58. Neel, J. V., Itano, H. A., and Lawrence, J. S., *Blood* **3**, 434 (1953).
59. Zuelzer, W. W., and Kaplan, E., *Blood* **9**, 1047 (1954).
60. Chernoff, A. I., Minnich, V., and Chongchareonsuk S., *Science* **120**, 605 (1954).
61. Lie-Injo Luan Eng, *Nature* **176**, 469 (1955).
62. Sturgeon, P., Itano, H. A., and Bergren, W. R., *Blood* **10**, 396 (1955).
63. Chernoff, A. I., Minnich, V., NaNakorn, S., Tuckinda, S., Kashemsant, C., and Chernoff, R. R., *J. Lab. Clin. Med.* **47**, 455 (1956).
64. Neel, J. V., *Blood* **7**, 467 (1952).
65. Lehmann, H., *Eugenics. Rev.* **46**, no. 2 (1954).
66. Roberts, D. F., and Lehmann, H., *Brit. Med. J.* **519**, (1955).
67. Lehmann, H., *Comm. Ve Intern. Congress of Blood Transfusion*, Paris, 1954.
68. Edington, G. M., and Lehmann, H., *Trans. Roy. Soc. Trop. Med.* **48**, 332 (1954).
69. Schneider, R. G., *J. Lab. Clin. Med.* **44**, 133 (1954).
70. Portiers, A., Cabannes, R., Massonnet, J., and Duval, J., *Algérie Med.* **59**, 563 (1954).
71. Jacob, G. F., *Brit. Med. J.* 521, (1955).
72. Stewart, J. W., and Mac Iver, J. E., *Lancet* **ii**, 23 (1956).
73. White, J. C., and Beaven, G. H., *J. Clin. Path.* **7**, 175 (1954).
74. Bird, G. W. G., Lehmann, H., and Mourant, A. E., *Transact. Roy. Soc. Trop. Med. and Hyg.* **49**, 399 (1955).
75. Cabannes, R., Unpublished observations.
76. Lehmann, H., Story, P., and Thein, H., *Brit. Med. J.* 544, (1956).
77. Aksoy, M., Bird, G. W. G., Lehmann, H., Mourant, A. E., Thein, H., and Wichremasinghe, R. L., *J. Physiol.* **130**, 56P (1955).
78. Tiagi, G. K., Haldar, P. K., and Laka, P. N., *Indian J. Med. Sci.* **3**, 745 (1954).
79. Minnich, V., Na-Nakorn, M. S. S., Chongchareonsuk, S., and Kochaseni, S., *Blood* **9**, 1 (1954).
80. Lie-Injo Luan Eng, cited by Singer (5).
81. Silverberg, J. H., and Shotton, D., *New England J. Med.* **245**, 688 (1951).
82. Perabo, F., *Helv. paediatr. Acta.* **9**, 339 (1954).
83. Römer, M. A., and Dörken, H., *Med. Klin.* 703, (1953).
84. Nuyken, G., *Med. Klin.* 1955 (1954).
85. Matoth, Y., Shamir, Z., and Freundlich, E., *Blood* **10**, 176 (1955).
86. Banks, L. O., and Scott, R. B., *Pediatrics* **11**, 622 (1953).
87. Schwartz, H., and Spaet, T. H., *Clin. Res. Proc.* **III**, 51 (1955).
88. Tuttle, A. H., *J. Lab. Clin. Med.* **47**, 811 (1956).
89. Bird, G. W. G., and Lehmann, H., *Man* **56**, 1 (1956).

# Some Aspects of Bile Pigment Metabolism

*Rudi Schmid*

**I**N SPITE OF IMPRESSIVE ADVANCES OVER THE PAST FEW years, knowledge of the pathways and mechanisms of hemoglobin degradation is still incomplete. The magnitude of the problem can be judged from the voluminous bibliography (1), and the present discussion is intended to emphasize only certain aspects where recent findings have led to some modification of previous concepts.

## HEMOGLOBIN

The average life span of the normal human erythrocyte is 120 days (2). At the end of this period, the cells are destroyed, and the hemoglobin is broken down. Of the three hemoglobin constituents, the iron is almost completely reutilized for the formation of other iron-containing compounds (3), and the liberated globin is degraded and returned to the body pool of amino acids (2). On the other hand, the protoporphyrin is not preserved, but the porphyrin ring is opened, and the breakdown products are excreted (4,5). The exact nature and sequence of the individual steps in hemoglobin catabolism are still a matter of controversy.

Some investigators have assumed that hematin is formed as the first intermediate, and according to this view, the initial step would consist in the splitting of the iron-protoporphyrin complex from globin (6-8). This concept seems to be strongly supported by a number of studies, which have shown that hematin is readily converted to bile pigments (9-11). Recent experiments employing labeled hematin have confirmed these observations (12). In conditions associated with intravascular hemolysis (13,14), as well as in cases of severe liver disease (8,15), hematin is sometimes present in the serum in the form of methemalbumin (14). Under normal conditions, however, hematin cannot be demonstrated in the blood, probably because the pigment breakdown occurs chiefly inside the reticuloendothelial cells.

The further degradation of hematin was believed to proceed through loss of

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the trivalent iron leading to the formation of protoporphyrin IX $\alpha$  as a second intermediate (8). This step, however, is not supported by experimental evidence; in fact, it has been shown that intravenous injection of protoporphyrin and hematoporphyrin does *not* lead to increased bile pigment excretion (16,17), and recent studies, which demonstrated a very low-grade conversion of labeled protoporphyrin to stereobilin (18) do not provide an unequivocal answer, since it is conceivable that a small fraction of the injected protoporphyrin may first have been converted to hematin before being broken down to bile pigments (18, 19). In vitro, Kench and co-workers have failed to observe bile pigment formation by coupled oxidation of protoporphyrin and *l*-ascorbic acid, whereas under the same conditions, positive results were obtained with hematin, methemalbumin, hemoglobin, and methemoglobin (20). These findings suggest the possibility that the presence of a divalent or trivalent iron may be essential for the cleavage of the protoporphyrin ring (20).

An alternative route for the breakdown of hemoglobin to bile pigments has been proposed by Lemberg and his school (21). In vitro, the coupled oxidation of hemoglobin and ascorbic acid was found to result in choleglobin, a green bile pigment-iron-globin complex which, upon treatment with dilute acetic acid, yielded biliverdin and free iron (21). Choleglobin-like pigments have been demonstrated in normal rabbit erythrocytes (22) and in stored human red cells (23), as well as in red cells of rabbits treated with phenylhydrazine (24). It has been suggested that the so-called "easily split-off iron" of the erythrocyte may actually represent that portion of the red cell iron which makes part of the choleglobin (25).

The determination of choleglobin-like compounds in red cells is exceedingly difficult because of the large excess of hemoglobin present, and considerable controversy has arisen over the question of whether or not these green pigments may simply represent artifacts which are of little significance under in vivo conditions (20,26-28).

## BILE PIGMENTS

### BILIVERDIN

Whatever the principal route of hemoglobin catabolism in the mammalian organism may be—with hematin or with choleglobin as intermediates—it appears most likely that biliverdin is the first bile pigment formed (21). The bile of many species of amphibia and of birds contains only biliverdin (29); on the other hand, the bile of most mammals, including man, contains predominantly bilirubin (21). In the human body, biliverdin is apparently rapidly reduced to bilirubin, and under in vitro conditions such reduction has been demonstrated in a variety of tissues (30, 31). Biliverdin is not detectable in normal human plasma, but it frequently accompanies bilirubin in the serum of patients with complete obstruction of the common bile duct, cirrhosis, and hepatitis (32).

## BILIRUBIN

The principal bile pigment released from the reticuloendothelial tissue into the plasma is undoubtedly bilirubin. In the serum it is bound to albumin (33), which serves as a carrier, since free bilirubin is water insoluble at the *pH* of the blood (34). In the liver, bilirubin is conjugated with two mols of glucuronic acid (35,36), which renders it water soluble and hence suitable for excretion in the bile (Fig. 1). In addition to bilirubin diglucuronide, normal human bile also probably contains small amounts of bilirubin monoglucuronide (35) and traces of other diazo-positive pigments (37,38). In vitro experiments have shown that the conjugation of bilirubin with glucuronic acid can be achieved with a system containing liver microsomes and uridine diphosphate glucuronic acid. The azo derivatives of the synthesized bilirubin glucuronide have been separated and identified by chromatographic methods (39). In vivo, the liver is undoubtedly the main site of bilirubin conjugation, and it appears doubtful whether other organs, such as the kidneys, contribute significantly to the conjugation of bilirubin. It has been observed that after hepatectomy, dogs are unable to form and excrete bilirubin glucuronide (38).

In hemolytic jaundice and in congenital nonhemolytic hyperbilirubinemia (Gilbert's disease), the amount of pigment produced appears to exceed the capacity of the liver to conjugate and hence to excrete bilirubin, so that retention of free, nonconjugated bilirubin occurs in the serum (Fig. 2). In this type of jaundice, the van den Bergh reaction is predominantly indirect, and no bilirubinuria occurs, a finding which has long served to distinguish hemolytic from obstructive jaundice (8). On the other hand, in jaundice due to extrahepatic obstruction or to liver injury, predominantly affecting the excre-

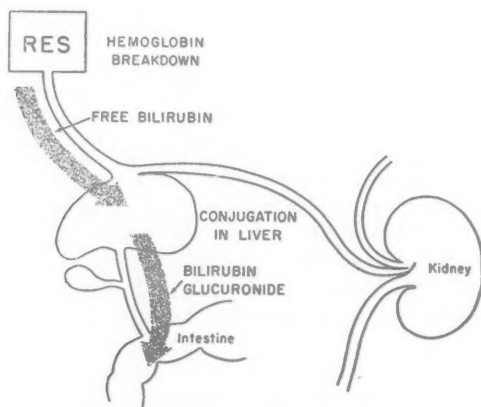


Fig. 1. Normal bilirubin metabolism.

tory capacity, conjugated bilirubin gains access to the blood where it can be recognized by giving a predominantly direct type of van den Bergh reaction. Electrophoretically, conjugated bilirubin also migrates with albumin (33), but probably because of its water solubility it is readily excreted in the urine (Fig. 3). It should be emphasized that in jaundiced serum, the direct van den Bergh reaction does not necessarily reflect the exact amount of conjugated bilirubin present, because a number of extrinsic variables, such as the concentration of bile acids, urea, and citrate in the serum have a significant effect upon the type of reaction curve obtained (1,40-44). The difference between the direct and the indirect van den Bergh reaction is largely determined by the difference in polarity of the two types of bilirubin, and factors which increase the solubility of nonconjugated bilirubin tend to result in a higher value for the direct-reacting fraction.

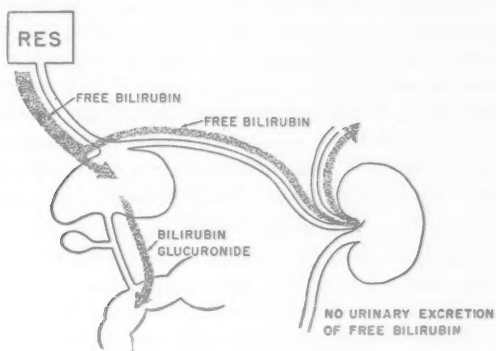


Fig. 2. Bilirubin metabolism in hemolytic jaundice (retention jaundice).

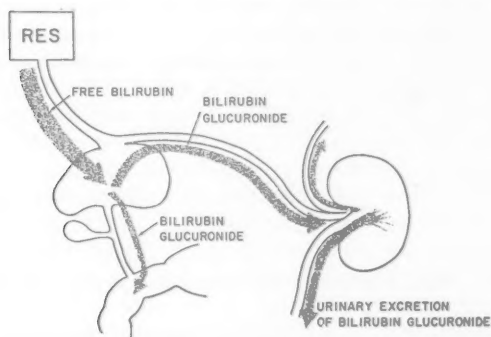


Fig. 3. Bilirubin metabolism in hepatogenous jaundice (regurgitation jaundice).

## HEME BREAKDOWN

Attempts to correlate the bilirubin excretion with the rate of hemoglobin breakdown as determined by other methods have shown that 80 to 100 per cent of the degraded hemoglobin can be accounted for as bile pigments (45). Most of these studies were carried out before the advent of isotopes, but the use of tracer technics has not invalidated these earlier experiments, although it has led to the discovery that certain quantitative aspects of bile pigment formation are a good deal more complicated than had previously been assumed. For instance, in the earlier studies, the portion of bile pigments derived from heme proteins other than hemoglobin had not received adequate consideration, mainly because of the lack of information regarding the concentration and turnover rate of these chromoproteins in the organism. By using isotopically labeled glycine, it has been found that a substantial portion of the excreted bile pigment is normally derived from sources other than the hemoglobin of mature circulating erythrocytes (46). In normal man, this fraction amounts to at least 10 to 20 per cent of all the bile pigments formed, and in disease states such as pernicious anemia or erythropoietic (congenital) porphyria, it may be much larger (47-49). London has discussed some of the possible sources of this bile pigment fraction (5) which may include myoglobin, catalase, cytochromes, and peroxidases, as well as immature erythrocytes which may be destroyed in the marrow or shortly after their release into the circulation.

There can be little doubt that in most instances, bilirubin and the pigments and chromogens resulting from its bacterial reduction in the intestines, represent the most important end product of heme degradation. There are, however, occasional cases where a significant discrepancy seems to exist between the amount of heme broken down and the bile pigments recovered (1,50-52). This is most obvious in patients suffering from severe congenital nonhemolytic jaundice of the type described by Crigler and Najjar (53) and Rosenthal (54). Patients with this syndrome appear to be almost unable to conjugate bilirubin with glucuronic acid (55), and this seems to be the reason for their defect in bilirubin excretion. Bile from such cases, although otherwise normal, was found to be almost totally devoid of bile pigments, and no bilirubin could be demonstrated in the urine (35). In spite of this nearly complete pigment retention, the concentration of nonconjugated serum bilirubin remained almost stationary, which is very much contrary to what one would expect, if bilirubin were the sole breakdown product of hemoglobin. It is obvious that alternative pathways of heme catabolism must exist, which lead to metabolites other than bilirubin. These may be dipyrrols or monopyrrols which are excreted without being detected, mainly because satisfactory methods for their demonstration are not available.

In the feces, dipyrrolic compounds belonging to the group of bilifuscin, mesobilifuscin, and pentdyopent, have been identified and isolated (32,56-58),



and pentdyopent has occasionally been found in pathological urines (59,60). However, it has not yet been established with certainty whether these compounds are actually metabolites resulting from intestinal degradation of bilirubin and other bile pigments, or whether they are artifacts in the sense that they are produced in the course of the extraction procedures to which the fecal material is subjected (21). With (61), and Lups and Meijer (62) have suggested that dipyrrols of the bilifuscin type may occur in normal and jaundiced sera, but direct evidence to support their claims has not been obtained. In vitro, exposure of hemoglobin to hydrogen peroxide (63) or coupled oxidation with fatty acids (64) has been shown to yield dipyrrolic compounds, but to date there is no indication that such mechanisms are of significance in vivo (29).

Recently, however, a congenital and familial hemolytic syndrome with erythrocyte inclusion bodies has been discovered where part of the hemoglobin appears to be degraded to dipyrrols, rather than to the usual tetrapyrrols (65,66). In these patients, large amounts of pigments belonging to the bilifuscin-mesobilifuscin group are present in the plasma and are excreted in the urine, giving it a dark brown-to-black appearance. Transfusion of erythrocytes from such a patient into a normal recipient was followed in the latter by urinary excretion of the same dark pigments. This observation seems to indicate that the pigment abnormality is the result of a defect in the red cells. Similar metabolic defects leading to degradation of heme to metabolites other than bilirubin will undoubtedly be recognized in the future.

## REFERENCES

1. With, T. K., *Biology of Bile Pigments*, Copenhagen, Arne Frost-Hansen, 1954.
2. Wintrobe, M. M., *Clinical Hematology*, Philadelphia, Lea, 1956.
3. Moore, C. V., and Dubach, R., *J.A.M.A.* **162**, 197 (1956).
4. Hawkins, W. B., and Johnson, A. C., *Amer. J. Physiol.* **126**, 326 (1939).
5. London, I. M., *Bull. N. Y. Acad. Med.* **30**, 509 (1954).
6. Nencki, M., and Zaleski, J., *Z. f. physiol. Chem.* **30**, 384 (1900).
7. Eppinger, H., *Die Hepato-lienalen Erkrankungen*, Berlin, Springer, 1920.
8. Watson, C. J., in *Handbook of Hematology*, H. Downey, (Ed.) London, Hamilton, 1938.
9. Brugsch, T., and Retzlaff, K., *Z. Exp. Path. Ther.* **8**, 645 (1912).
10. Mann, F. C., Sheard, C., Bollmann, J. L., and Baldes, E. J., *Amer. J. Physiol.* **76**, 306 (1926).
11. Pass, I. J., Schwartz, S., and Watson, C. J., *J. Clin. Invest.* **24**, 283 (1945).
12. London, I. M., *J. Biol. Chem.* **184**, 373 (1950).
13. Bingold, K., *Z. Klin. Med.* **97**, 257 (1923).
14. Fairley, N. H., *Quart. J. Med.* **10**, 95 (1941).
15. Schumm, O., *Hoppe Seyl. Z.* **97**, 32 (1916).
16. Watson, C. J., Pass, I. J., and Schwartz, S., *J. Biol. Chem.* **139**, 583 (1941).
17. Bénard, H., Gajdos, A., Polonovski, M., and Tissier, M., *Presse Méd.* **56**, 37 (1948).
18. London, I. M., Yamasaki, M., and Sabella, G., *Fed. Proc.* **10**, 217 (1951).
19. London, I. M., Personal communication.
20. Kench, J. E., Gardikas, C., and Wilkinson, J. F., *Biochem. J.* **47**, 129 (1950).

21. Lemberg, R., and Legge, J. W., *Hematin Compounds and Bile Pigments*, New York, Interscience Pub., 1949.
22. Lemberg, R., and Legge, J. W., *Aust. J. Exp. Biol. Med. Sci.* **20**, 65 (1942).
23. Gajdos, A., and Tirprez, G., *Compt. rend. Soc. Biol.* **139**, 545 (1945).
24. Kiese, M., and Seipelt, L., *Arch. f. Exper. Path. U. Ther.* **200**, 648 (1943).
25. Barkan, G., and Walker, B. S., *J. Biol. Chem.* **131**, 447 (1939).
26. Gardikas, C., Kench, J. E., and Wilkinson, J. F., *Nature* **161**, 607 (1948).
27. Lemberg, R., *Nature* **163**, 97 (1949).
28. Gardikas, C., Kench, J. E., and Wilkinson, J. F., *Biochem. J.* **46**, 85 (1950).
29. Gray, C. H., *The Bile Pigments*, London, Methuen, 1953.
30. Lemberg, R., and Wyndham, R. A., *Biochem. J.* **30**, 1147 (1936).
31. Baumgärtel, T., *Z. Ges. Exptl. Med.* **112**, 459 (1943).
32. Watson, C. J., *New England J. Med.* **227**, 665 (1942).
33. Klatzkin, S., and Bungards, L., *J. Clin. Invest.* **35**, 537 (1956).
34. Overbeek, J. Th. G., Vink, C. L. J., and Deenstra, H., *Réc. Trav. Chim. Pays-Bas.* **74**, 81 (1955).
35. Schmid, R., *Science* **124**, 76 (1956).
36. Billing, B. H., and Lathe, G. H., *Biochem. J.* **63**, 6P (1956).
37. Cole, P. G., Lathe, G. H., and Billing, B. H., *Biochem. J.* **57**, 514 (1954).
38. Schmid, R. (to be published).
39. Schmid, R., Hammaker, L., and Axelrod, J. *Arch. Biochem. Biophys.* (in press).
40. Schelagurov, A. A., *Chem. Zentralblatt* **103**, 2194 (1937).
41. Adler, A., and Strausz, L., *Z. f. d. Ges. Exper. Med.* **44**, 1 (1925).
42. Bungenberg de Jong, W. J. H., *Dtsch. Arch. f. Klin. Med.* **190**, 229 (1943).
43. Barron, E. S. G., *Medicine* **10**, 77 (1931).
44. Thompson, H. E., and Wyatt, B. L., *Arch. Internal Med.* **61**, 481 (1938).
45. Cruz, W. O., Hawkins, W. B., and Whipple, G. H., *Amer. J. Med. Sci.* **203**, 848 (1942).
46. London, I. M., West, R., Shemin, D., and Rittenberg, D., *J. Biol. Chem.* **184**, 351 (1950).
47. Gray, C. H., Neuberger, A., and Sneath, P. H. A., *Biochem. J.* **47**, 87 (1950).
48. London, I. M., and West, R., *J. Biol. Chem.* **184**, 359 (1950).
49. London, I. M., West, R., Shemin, D., and Rittenberg, D., *J. Biol. Chem.* **184**, 365 (1950).
50. Ottenberg, R., *J. Mount Sinai Hospital* **9**, 937 (1943).
51. With, T. K., *Acta Med. Scand.* **123**, 166 (1946).
52. Deenstra, H., *Ann. de Méd.* **51**, 685 (1950).
53. Crigler, J. F., and Najjar, V. A., *Pediatrics* **10**, 169 (1952).
54. Rosenthal, I. M., Zimmerman, H. J., and Hardy, N., *Pediatrics* **18**, 378 (1956).
55. Schmid, R., Axelrod, J., Hammaker, L., and Rosenthal, I. M., *J. Clin. Invest.* (in press).
56. Siedel, W., *Ber.* **77A**, 21 (1944).
57. Meldolesi, G., Siedel, W., and Möller, H., *Hoppe Seyl. Z.* **259**, 137 (1939).
58. Stich, W., *München. Med. Wchnschr.* **92**, 1276 (1950).
59. Bingold, K., *Deut. Arch. Klin. Med.* **177**, 230 (1935).
60. Hulst, L. A., and Grotepass, W., *Klin. Wchnschr.* **15**, 201 (1936).
61. With, T. K., *Nature* **158**, 310 (1946).
62. Lups, S., and Meijer, F. G. D., *Acta Med. Scand.* **126**, 85 (1946).
63. Bingold, K., *Klin. Wschr.* **20**, 331 (1941).
64. Haurowitz, F., Schwerin, P., and Yensen, M. M., *J. Biol. Chem.* **140**, 353 (1941).
65. Schmid, R., Brecher, G., and Williams, G. Z., and Clemens, T., Jr., *Proc. Sixth International Congress of Hematology* (Paper #540), Boston, 1956.
66. Schmid, R., and Siedel, W. (unpublished observations).

# Standardization in Clinical Chemistry

I. D. P. Wootton

WHEN A NUMBER OF DIFFERENT LABORATORIES all examine a single specimen, say, of blood plasma, and they all determine a particular constituent, then in general, they will not all return the same result. If the different results are reasonably close, the method can be considered to be well standardized. Frequently, however, it is apparent that standardization is not sufficiently good, so that the clinical interpretation depends on where the analysis was done. In addition, difficulties are encountered when a patient is transferred from one hospital to another. Moreover, insufficient standardization may cause quite wrong interpretations to be drawn from survey data. As an example, the data on normal hematologic values in Table 1 can be cited. When the English values for 1936, are compared with the U. S. values, it appears that English subjects had a lower total hemoglobin, although the red cell counts in both countries were the same. The English survey was done with hemoglobin estimated by the Haldane method and the conversion factor into grams of hemoglobin was subsequently shown to be incorrect (King *et al.*, 1947). More recent surveys with conversion factors in better international agreement have shown that the apparent anemia of the Old World is an illusion.

In describing the progress which has been made toward standardization, reference will largely be confined to experience in Great Britain. This is not intended to be an insular attitude but is taken only for convenience, since the evolution of the subject is following parallel lines in several countries.

## INVESTIGATION OF THE PROBLEM

### NORMAL VALUES

During the years around 1950, our laboratory became interested in the range of normal values for various blood constituents. An accurate knowledge of

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It is a pleasure to record my debts to Prof. E. J. King for his constant encouragement, to my colleagues in the various committees and commissions engaged in this work, and to the many analysts who provided the raw material of the investigations.

Table 1. HEMATOLOGIC VALUES OF NORMAL MALE SUBJECTS

Source	Red cell count (millions/ml.)	Total hemoglobin (Gm./100 ml.)	Mean corp. hemoglobin concentration (%)	References
England (1935)	5.4	14.6	31.3	Price-Jones <i>et al.</i> (1935)
U.S. (1942)	5.4	16.0	34.0	Wintrobe (1942)
England (1944)	5.5	16.1	34.0	Macfarlane and O'Brien (1944); King <i>et al.</i> (1944)

the normal variation is essential for the proper interpretation of results, and we therefore examined blood specimens from 50-100 normal subjects. An interesting finding emerges; only about half the constituents examined were distributed as a classic "normal" distribution. The remaining constituents (except one) were distributed in a skewed fashion but could be closely fitted by a normal log curve. By this means we were able to calculate precise values which would embrace, say, 80 or 98 per cent of the results found in the normal persons in our population (Wootton *et al.*, 1951; Wootton and King, 1953).

#### BIOCHEMICAL TRIALS IN BRITAIN

The precise and objective limits to normal values which were set by the previous investigation differed in several respects from the limits which were normally accepted both by ourselves and by other workers. It soon became clear that a barrier to the general acceptance of any set of normal values lay in the interhospital differences in results. To investigate this, we distributed a specimen of whole blood and a synthetic aqueous solution, with a request that these specimens should be analyzed for certain named constituents. Results were obtained from about 20 laboratories for the first specimen and from about 50 laboratories for the second (Wootton and King, 1953).

The values obtained demonstrated that the analyses chosen were far from standardized and that even with such common determinations as blood urea or inorganic phosphate, the situation was far worse than we had imagined. Similar findings have been reported by other investigators, such as Belk and Sunderman, 1947; Sherry and Cebel, 1949; and Henry, 1952.

#### THE INTERNATIONAL BIOCHEMICAL TRIAL

This trial was organized by the Commission on Clinical Chemistry of the International Union of Pure and Applied Chemistry and the Committee of the International Federation of Clinical Chemistry. We are grateful to the Union for a grant to cover the finances of this trial; detailed results have already been reported (Wootton, 1956).

The objects of the trial were twofold. Firstly, we wished to find out whether this low level of standardization was universal among all the countries taking

part in the trial. Secondly, there was a variation on the usual pattern for conducting the trial, in that two samples of freeze-dried serum were circulated instead of a single sample. The second sample was an accurate dilution of the first and was only 70 per cent as concentrated. It was therefore possible to determine whether the ratio of the two results provided by a laboratory was more accurate than the absolute values.

The results showed that all the countries concerned were very similar in the variation of results, i.e., France, Italy, Netherlands, South Africa, Scandinavia, United States, United Kingdom, and Yugoslavia. It was also shown that the ratio of the two results was always less variable than the absolute values. The conclusion is that an immediate improvement can be made by arranging the analytic procedure so that an unknown is determined by comparing it with a known standard.

Finally, although with most constituents examined the mean value returned by all the laboratories in each country was very close to the grand mean of all estimations, indicating no systematic difference in level between countries, this did not hold for glucose. The grand mean of the glucose results was 129 and 93 mg./100 ml. for the two samples. The U. S. mean, on the other hand, for the same samples, was 103 and 72 mg./100 ml. At first sight, this might be taken to be a reflection of the methods used, by postulating that the U. S. generally reported "true sugar" while most of the remainder reported "total reducing substances." Reference to the report forms does not support this view and there seems to be no ready explanation.

### SUGGESTED REMEDIES

#### INTERNAL CHECKING

A great deal can be done by the laboratory itself to maintain an acceptable level of precision. A convenient scheme of internal checking which has been in use for several years has recently been described (King and Wootton, 1956). A control sample of the constituent to be analyzed is included with each batch; the value found is plotted on a control chart (Wernimont, 1946; Levey and Jennings, 1950; Henry and Segalove, 1952). If the value found is further from the true answer than the error allowed, then the batch must be reanalyzed. The errors allowed for certain common analyses in our own laboratory are given in Table 2; they may at first sight appear to be unnecessarily lax, but they are the result of considerable investigation and are, we believe, representative of good clinical analytic standards.

There is one important point in constructing an internal check of this kind. It is essential that the analyst who is performing the determinations should not know the answer expected for the control solution: this does not imply any suspicion of dishonesty on the part of the analyst but is an essential precaution to avoid the powerful unconscious bias which otherwise operates to make the result of the control solution better than it otherwise should be.

Table 2. MAXIMUM ERRORS ALLOWED IN CONTROL SOLUTIONS

<i>Solution</i>	<i>Error</i>
Sodium	$\pm 2$ mEq./l.
Potassium	$\pm 0.2$ mEq./l.
Chloride	$\pm 1.5$ mEq./l.
Bicarbonate	$\pm 1.0$ mEq./l.
Urea	$\pm 10\%$ of value found
Glucose	$\pm 10\%$ of value found

## CERTIFIED SAMPLES

There seems to be a very good case for every laboratory carrying out an additional check by analyzing a certified sample at regular intervals. Such samples may be synthetic solutions. Alternatively, they may be of such material as freeze-dried serum, which has been examined by one or more reference laboratories, who can be expected to provide analyses of a higher order. This condition leads to difficulties with material samples as opposed to synthetic solutions, although it is generally accepted that natural samples provide a much better test material. Such certified samples are also required in large numbers, which means in practice that the cooperation of commercial organizations is necessary. In spite of these difficulties, several countries have started schemes which use natural material; in England, a joint committee of the Association of Clinical Pathologists and the Association of Clinical Biochemists is responsible for the analyses and we have been most fortunate in having the services of Glaxo Laboratories, Ltd., and C. Davis Keeler, Ltd., who, respectively, prepare and distribute the samples.

An older, but more specialized, scheme is the Medical Research Council Hemoglobin Standards. This consists of the dispatch, once every 2 months, of a sample of blood, suitably preserved, for the checking of hemoglobinometry. The blood is certified for hemoglobin content at the Postgraduate Medical School of London by spectrophotometry and iron analysis and is distributed by Keelers. The scheme has been regularly in operation since 1951. Distribution is now worldwide, and the samples have proved of great value in standardizing all the many different methods of hemoglobin determination.

## FUTURE DEVELOPMENTS

It seems very likely that many more laboratories will organize their own systems of quality control within their own walls. There is also every indication that more countries are about to commence schemes of issuing certified samples. In this field it seems essential that there should be international co-operation to avoid the possibility that different national schemes will be operated which do not agree with one another. In this respect, comparisons have already been made between samples from the British M R C Hemo-

globin Standards and samples of the Drabkin cyanmethemoglobin standards distributed in the U. S., and it is very satisfactory to report that the standards correspond within about 1 per cent. However, as the results of the international trial show in glucose estimations, such a fortunate result will not necessarily emerge, and steps should be taken regularly to exchange certified samples between countries. It is also worth considering whether it would not be more efficient to operate very large schemes to cover a number of countries. The financial undertaking would need careful study.

Finally, much of the scatter in results has been attributed by some people to the use of different analytic methods. It has been suggested that some measure of standardization of method would be an advantage. In my view, this approach has only a limited value, since a method suitable for a large hospital department may be quite inappropriate for a small laboratory. It is also, to some extent, irrelevant, because reports are made of the concentration, in the specimen, of defined chemical substances, and the value obtained cannot be satisfactory if it depends on the analytic method which is used. Moreover, we have found that certifying laboratories of a high professional standard agree surprisingly well, although they do not all use the same methods. No doubt as methods improve, this cause of disagreement will become less important.

#### REFERENCES

1. Belk, W. P., and Sunderman, F. W., *Amer. J. Clin. Path.* **17**, 853 (1947).
2. Henry, R. J., *Clin. Chem.* **4**, 4 (1952).
3. Henry, R. J., and Segalove, M., *J. Clin. Path.* **5**, 305 (1952).
4. King, E. J., Gilchrist, M., and Matheson, A., *Brit. Med. J.* **i**, 250 (1944).
5. King, E. J., Gilchrist, M., Wootton, I. D. P., Donaldson, R., Sisson, R. B., Macfarlane, R. G., Jope, H. M., O'Brien, J. R. P., Peterson, J. M., and Strangeways, D. H., *Lancet* **ii**, 789 (1947).
6. King, E. J., and Wootton, I. D. P., *Microanalysis in Medical Biochemistry*. (3rd ed.) London, Churchill, 1956.
7. Levey, S., and Jennings, E. R., *Amer. J. Clin. Path.* **20**, 1059 (1950).
8. Macfarlane, R. G., and O'Brien, J. R. P., *Brit. Med. J.* **i**, 248 (1944).
9. Price-Jones, C., Vaughan, J. M., and Goddard, H. M., *J. Path. Bact.* **40**, 503 (1935).
10. Sherry, H. E., and Cebel, J., *Bull. U. S. Army Med. Dep.* **9**, 799 (1949).
11. Wernimont, G., *Industr. Engng. Chem. (Anal.)* **18**, 587 (1946).
12. Wintrobe, M. M., *Clinical Haematology* (2nd ed.). London, Kimpton; Philadelphia, Lea, 1942.
13. Wootton, I. D. P., King, E. J., and Maclean Smith, J., *Brit. Med. Bull.* **7**, 307 (1951).
14. Wootton, I. D. P., and King, E. J., *Lancet* **i**, 470 (1953).
15. Wootton, I. D. P., *Clin. Chem.* **2**, 296 (1956).

# Ultramicro Methods and Standardization of Equipment

M. C. Sanz

THE PRESENT SITUATION OF ULTRAMICROANALYSIS is comparable to that of analytic chemistry about 60 years ago. Every analyst developed his own equipment and methods, according to his abilities and his investigative problems. A multitude of very different apparatus and methods was the result of these different approaches to the problem. In contrast to this development, the microanalytic era started about 35 years ago in Vienna and Graz. A few outstanding men like Emich, Feigl, Pregl, and others, developed micromethods and soon reached a very high analytic level. The special equipment was soon adopted all over the world.

Ultramicromethods are now being developed in many laboratories and in very different fields. One of the most important pioneers was certainly Linderström-Lang whose outstanding achievements in cellular chemistry I need not recall here. Another pioneer, Paul L. Kirk has contributed a great deal to the ultramicro field, mainly in inorganic analysis both during and since his work on the Manhattan Project.

A large variety of apparatus and methodical approaches is being described in different types of journals. I am not going to give a general review of the ultramicromethods, but will limit myself to microanalytic problems in biochemistry and in this field mainly to clinical chemistry. I would like to discuss our line of approach to the problem of introducing ultramicromethods into the routine laboratory.

The reasons for doing so are numerous: (1) To save technicians. Any large hospital needs, besides the ordinary routine laboratory, a special micro or ultramicro laboratory for pediatrics, ophthalmology, and all the cases where the laboratory receives samples too small for analysis. (2) To save space. (3) To save reagents and glassware. (4) All research problems greatly benefit from a well-working and well-equipped ultramicro routine laboratory. Since in the routine laboratory the methods must be simple, we do not try to reach

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the ultimate limits that have been attained by others or that could be reached with our equipment. There have been some methods published for the determination of much smaller quantities than we use, but we have to restrict ourselves to safety limits permitting easy work.

The term "ultramicro methods" in biologic and clinical chemistry is referred to the volume of serum or blood used for a determination (i.e., up to 50  $\mu$ l.), rather than to the quantity of the determined constituent.

In case of need it is often very easy to determine smaller quantities than we do, simply by using a more sensitive buret or a smaller optical cuvet for the final determination.

### GENERAL CONSIDERATIONS CONCERNING WORK ON AN ULTRAMICRO SCALE

Quite a number of reactions will not turn out the usual way if one simply diminishes all the volumes proportionally. One of the main reasons for this paradox is the fact that the surface is relatively greatly enlarged with respect to the volume, due to creeping along wettable surfaces. Hence the use of water-repellent surfaces is imperative in many steps involved in ultramicro-analysis. We treat the glass surfaces with Desicote and use, to a large extent, many different plastics, especially thermoplasts as polyvinylchloride, polyethylene and its fluorinated derivatives, polyacrylics, and polyamids. Of great help also are paraffin and parafilm.

Since in all titrations the tip of the buret dips into the solution, it must end in a very fine capillary, the exterior of which must be water repellent in order to avoid creeping. We routinely use polyethylene tips attached to a glass capillary by polyvinylchloride capillary tubing. We have never succeeded in making a good seal between polyethylene and glass capillaries. Polyethylene tips have the advantage of being unbreakable and easily interchangeable. Using water-repellent surfaces, the requirements concerning the purity of reagents and cleanness of the glassware are the same as in macromethods, with one exception: the optical faces of the microcuvets, when using a fine light beam, must be rigorously clean, much cleaner than those of macrocuvets. Cleaning of the glassware, such as titration cups, test tubes, and centrifugation tubes is very often done by simply rinsing with water and wiping off with cellulose paper.

On the other hand, air-borne impurities like dust, ammonia, sodium, calcium, iron, and others, may constitute a major source of error. Freedom of dust in the working room is capital. No ventilation is preferable to any air-renewing system without dust filter.

The volumes are kept throughout as small as possible, avoiding work with highly diluted solutions. We even sometimes use more concentrated titration solutions than in ordinary methods, thus making the end point sharper.

We generally centrifuge rather than filter, which can be done very rapidly on small volumes, as the ultramicrocentrifuges easily attain 20,000 to 30,000 rpm. If possible the whole analysis is carried out in the same vessel, avoiding all transferring.

Generally speaking the titration methods are more accurate than colorimetric methods and often faster. Whenever it is advantageous we use titrimetric methods. No gravimetric methods are used, as an ultramicrobalance is very expensive and delicate to handle.

Two steps at least must be carried out accurately in any analysis: the sample delivery and the final measurement of the constituent. In colorimetry this means that in addition the final volume must be accurately adjusted as well. For this purpose one can use a volumetric flask or one can add precisely every solution in order to still have sufficient precision in the final volume. We usually follow the last way. Three ultramicro methods will be omitted from our discussion, being already in general use all over the world: flame-photometry, zone electrophoresis, and paperchromatography.

#### MATERIALS AND METHODS

All our pipets are of the self-adjusting capillary type, sealed in a glass tube (Fig. 1). They are made of glass or polyethylene. The latter are unbreakable and allow continuous pipetting without washing. The polyethylene used is of a carefully chosen quality, as most of the commercially available material is not suitable, due to impurities, uneven surfaces, or rapid crystallization.

The pipets are filled by suction and carefully emptied by blowing, which can be done by mouth through a fine rubber tubing or by means of a little

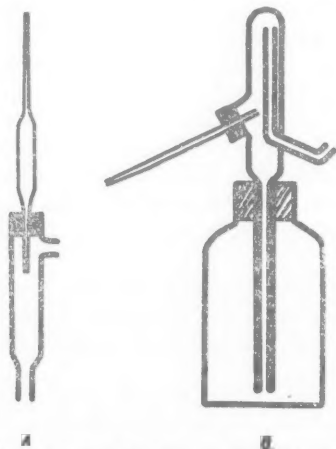


Fig. 1. A. Sample pipet. B. Reagent pipet.

plastic bottle. With the latter the action of the pipet is controlled by finger-tip pressure upon the lateral opening of the glass tube. The handling of these pipets is easy and rapid. The performance data are shown in Table 1.

When using this type of pipet three points are essential in order to get good results: (1) Empty slowly. (2) On filling, to always let the pipet "overflow." That means to let one or two drops fall off at the upper end. This will automatically wash out the pipet and assure a constant volume. (3) The pipets which have served for pipetting serum, plasma, or blood during the day are left overnight, filled with a fresh solution of 1% pepsine in 0.1N HCl. Thus an invisible film of protein is removed.

All the pipets are calibrated to "deliver" which is easily done by weighing

Table 1. PIPET-CALIBRATION DATA WITH HUMAN SERUM

5 $\mu$ l.	10 $\mu$ l.	20 $\mu$ l.	5 $\mu$ l.
5.05 mg. —2	10.14 mg. —4	20.34 mg. —1	5.150 mg. 6
5.03 —4	10.18	20.36 1	5.138 —6
5.04 —3	10.17 —1	20.30 —5	5.146 2
5.06 —1	10.19 1	20.38 3	5.128 —16
5.04 —3	10.19 1	20.36 1	5.135 —9
5.06 —1	10.13 —5	20.33 —2	5.156 12
5.06 —1	10.25 7	20.30 —5	5.158 14
5.09 2	10.15 —3	20.33 —2	5.127 —17
5.10 3	10.13 —5	20.40 5	5.140 —4
5.09 2	10.14 —4	20.38 3	5.160 16
5.06 —1	10.17 —1	20.40 5	
5.07	10.19 1	20.32 —3	
5.04 —3	10.24 6	20.34 —1	
5.08 1	10.21 3	20.38 3	
5.06 —1	10.20 2	20.32 —3	
5.07	10.13 —5	20.38 3	
5.09 2	10.24 6	20.39 4	
5.07	10.15 —3	20.30 —5	
5.05 —2	10.24 6	20.31 —4	
5.09 2	10.20 2	20.34 —1	
$\bar{x} = 5.07$ mg.	$\bar{x} = 10.18$ mg.	$\bar{x} = 20.35$ mg.	$\bar{x} = 5.144$ mg.
$s = \pm \sqrt{\frac{\sum(\bar{x} - x_1)^2}{n-1}}$			
$s = \pm 0.41\%$	$s = \pm 0.39\%$	$s = \pm 0.17\%$	$s = \pm 0.233\%$

Reproducibility of reagent pipets:

20  $\mu$ l. pipet: 20.21 mg., standard deviation  $\pm 0.124\%$

2.5  $\mu$ l. pipet: 2.51  $\mu$ l., standard deviation  $\pm 0.128\%$  (tested by optical method)

them filled with water and empty on a good analytical balance (sensitivity 0.01 mg.). The volumes that have been used by us range from 1 to 250  $\mu$ l.

The same type of capillary pipet is used for pipetting reagents. They are mounted on top of the reagent bottles and are filled by hand pressure. On pressing a second time they are automatically emptied, showing a reproducibility which is even better than that of the sample pipets (see Table 1). Thus every reagent has its own pipet which is always ready for use and never needs washing. The volumes of the reagent bottles are 50 or 100 ml., making 1000 to 5000 analyses possible without refilling. A reagent cannot be delivered otherwise than by its prescribed volume.

For the titration, the buret, the stirrer, and if needed the electrodes are stably mounted on a support and the titration vessel is lifted upwards by means of a mobile table (Figs. 2 and 3).

The proposed titration table is sturdy and moves smoothly. It has proved the best of many different constructions. The slope of the screw has 5 mm. per turn and remains stable in any position without fixation. The material of the table is of white polyvinylchloride, thus permitting the drilling of holes and the fixing of different titration vessels, like a cup glass electrode for potentiometric acid-base titrations.

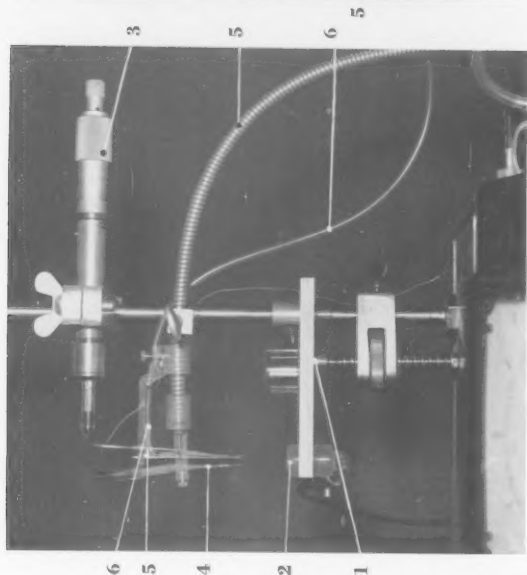
The stirrer is actioned by a little aquarium pump, working on the main A.C. line and built for continuous use. By means of a fine wire mounted in a flexible metal tube remaining rigid in all positions, the vibrations are transmitted to a glass rod coated with polyethylene. The fixation of the stirrer in any position is extremely easy. This stirrer is well suited for all stirring problems in ultramicroanalysis, like stirring in an open or a closed vessel or in a hanging drop.

The same aquarium pump may be used as a sucking and blowing unit for air or liquids, for example, wash water (Fig. 3). It is also used for filling and washing the glass electrode for the blood pH.

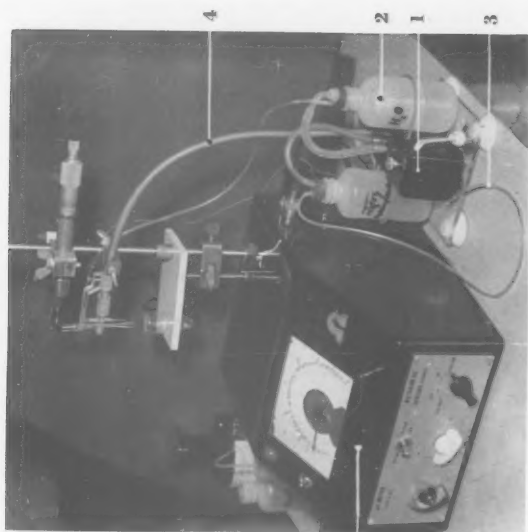
Figure 4 shows a new buret permitting titrations with very small volumes. The concentration of the titration liquid is adjusted so that 5 to 6  $\mu$ l. correspond to a normal value. The volumes are readable to 0.01  $\mu$ l. The total volume of the buret is 150  $\mu$ l., so that 25 to 30 titrations can be done without refilling. The volumes are read on a large dial with automatic zero adjustment between titrations. One revolution of the dial corresponds to 3  $\mu$ l.

The buret is of the micrometer-driven piston type, using neither mercury nor grease, and is of an extremely sturdy construction. The piston moves through a silicone gasket and does not touch the walls of the glass tube containing the titration liquid.

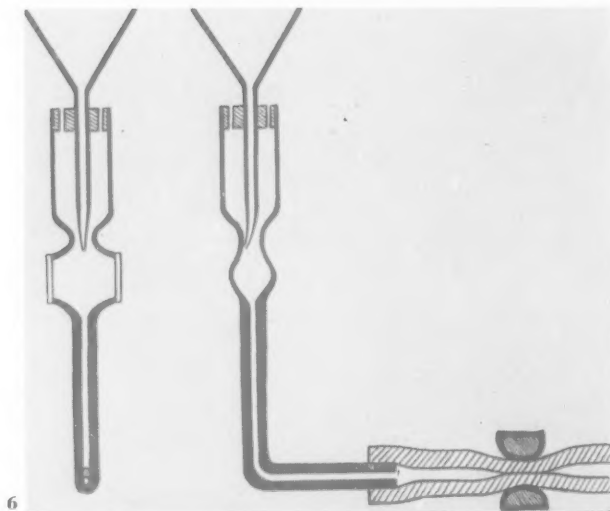
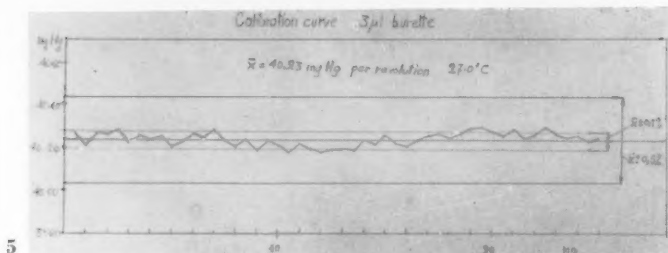
In a laboratory with reasonably constant temperature thermostabilization is not necessary. The precision of the buret depends on the precision of the piston, which is 2.764 mm. in diameter and can be made with an accuracy of



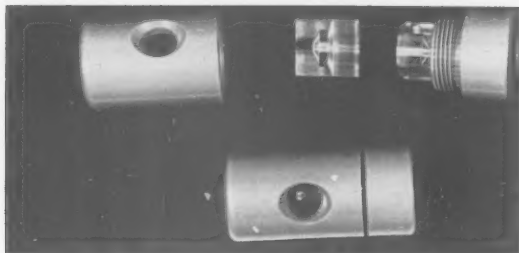
**Fig. 2.** Titration unit. (1) Moving titration table, (2), with cup glass electrode, (3) buret, (4) reference electrode, (5) stirrer, and (6) arrival of wash water.



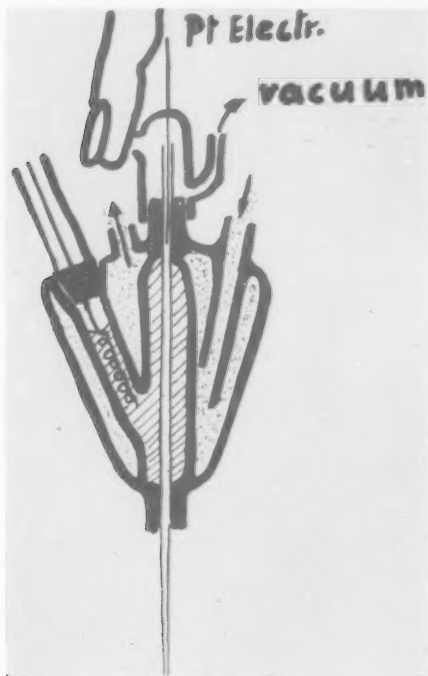
**Fig. 3.** Titration unit. (1) Aquarium pump, (2) reservoir for wash water, (3) aspiration tube, (4) vibrator tube, and (5) pH meter.



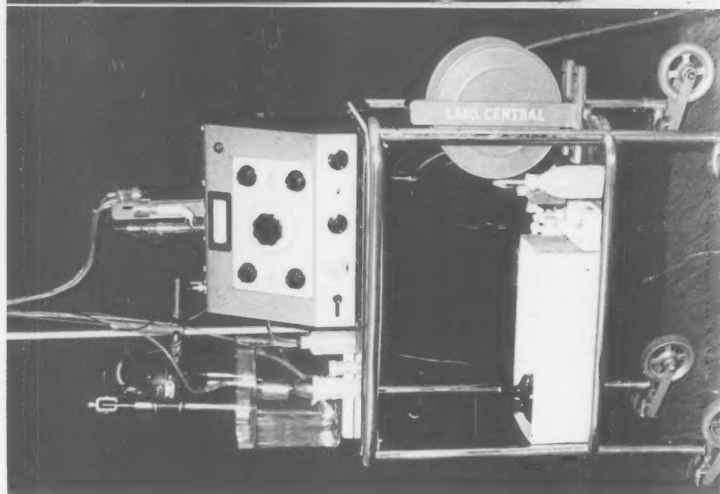
**Fig. 4.** Buret with dial for automatic zero adjustment. **Fig. 5.** Calibration curve for buret delivering 3  $\mu$ l. per revolution of the dial. **Fig. 6.** Optical cuvet for routine analysis with volumes of 0.5 ml.



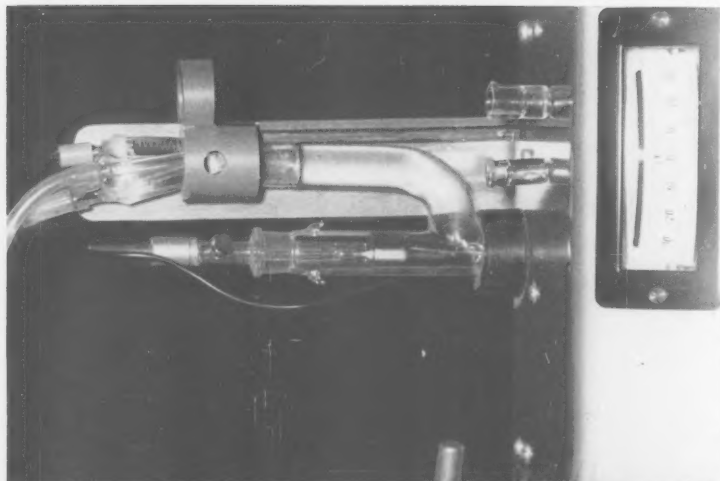
**Fig. 7.** Diffusion cell for the determination of ammonia.



**Fig. 8.** Capillary electrode for the measurement of the blood pH.



10



9

Fig. 9. Measuring unit for the blood pH. Fig. 10. Detail of the measuring unit. Glass electrode and calomel electrode in measuring position.



$\pm 0.01$  mm. in glass or  $\pm 0.002$  mm. in metal plated with rhodium (even more resistant to chemical attack than platinum).

Calibration is done with Hg of highest grade purity after thorough thermostabilization. Extreme care must be taken to reproduce the break-off of the mercury, which represents the major source of errors. Figure 5 shows a typical calibration curve. The reproducibility of the 3- $\mu$ l. volume (1 revolution of the dial) is within 0.1 per cent. On using 30  $\mu$ l. for a titration, the reproducibility is about 10 times better. The buret has two systematic errors: the mean value is not exactly 3  $\mu$ l. but 2.971, i.e. 99.0 per cent of the theoretical value, and the piston is thinner in the central part than in its ends. This error is found on all glass pistons and can considerably vary from one piston to another according to the care taken in its making. Although these differences in the diameter do not exceed 0.1 to 0.5 per cent, they may play a role in titrating with volumes of 1 or 2  $\mu$ l. where the errors can attain a high percentage. We hope to be able to eliminate both systematic errors by using metal pistons plated with rhodium.

On the same principle a buret, 30 times more sensitive, has been developed, one turn of the dial corresponding to 0.1  $\mu$ l., readable to 0.001  $\mu$ l. This buret needs strict thermostabilization and will be useful for special problems. We have not yet tested it thoroughly.

For colorimetry we have developed a new optical cuvet for a final volume of 0.5 ml. Figure 6 shows the principle of it. Through a fine polyethylene funnel the liquid enters the cell, which has a volume of 0.4 ml. The optical faces are made of glass or quartz and are sealed to the glass body with araldite. The outlet of the capillary of the cuvet is closed by a compressed silicone tubing. On filling the cuvet the air escapes through the holes drilled in the polyethylene plate holding the funnel. In order to avoid air bubbles *n*-caprylic alcohol in acetone and water are sucked through the cell after every 20 measurements. Emptying is done by pressing a button, which opens the silicone tubing and at the same time puts an aquarium pump into action. Thus the liquid is sucked into a waste bottle. As the cuvet is not moved, and all calibration curves are established with the same cuvet, its dimensions are not at all critical. This cuvet can easily be adapted to fit in any photometer. We use it with the Beckman Mod. C without any diaphragm. For routine use the cuvet is not cleaned, one solution being measured after the other. The liquid remaining in the cell is less than 1 per cent of the cell-volume.

For smaller volumes we have developed a new cylindrical cuvet which is very easily cleaned and holds about 70  $\mu$ l. The optical pathway is 10.00 mm. and the inner diameter 3 mm. The body is of Pyrex or Kel-F. The optical faces of glass or quartz are removable and thus easy to clean. Since in the last few days we have considerably transformed our unit neither a detailed description of it nor the performance data can be given.<sup>1</sup>

<sup>1</sup>The cuvet unit fitting the Beckman model DU will be manufactured by Beckman Instruments, Inc., Fullerton, Calif.

For the Kjeldahl determinations on the ultramicro scale we have developed a diffusion unit, made of polyacryl, easy to handle and assuring a tight seal without any grease (Fig 7).

The digestion is performed in a sealed tube by the method of Grunbaum (1) at 470° C. for 30 minutes. Our results with this procedure are excellent. On the other hand, we had some difficulties with the diffusion units of this author.

After digestion with  $\text{H}_2\text{SO}_4$ , the sample is diluted with  $\text{H}_2\text{O}$  and a 100- $\mu\text{l}$ . aliquot, containing 0.5 to 10  $\mu\text{g}$ . of nitrogen, is placed into the lower part of the unit. Forty  $\mu\text{l}$ . of saturated potassium hydroxyde are carefully introduced below the dilute acid solution, which makes an effective seal against losses of ammonia. First this part is put into the holder and then the upper part containing 50  $\mu\text{l}$ . of boric acid plus indicator (bromocresol green and methyl red). The boric acid will hang safely without running down. The holder is screwed firmly together and the unit placed in a nearly horizontal position in a slowly rotating disk provided with holes. Up to 20 determinations can be made at the same time. After 2½ hours the unit is unscrewed and the titration is carried out directly in the upper part, using the 3- $\mu\text{l}$ . buret (3  $\mu\text{l}$ . per revolution) filled with 0.01N HCl (1 revolution corresponds to 0.03  $\mu\text{mols}$  or 0.42  $\mu\text{g}$ . N).

#### DETERMINATION OF BLOOD pH

The difficulties encountered in the measurement of the blood pH are numerous. As we cannot yet measure in the blood stream—and there we would have to overcome two main difficulties, namely the stream potentials and the effect of traumatization of the vessel—we must try to approach as far as possible the ideal conditions. As a matter of fact one should be able to make the determination at the temperature and at the pressure of the patient, without adding any anticoagulant, as most of them are known to influence the pH, avoiding all contact with air, and as quickly as possible. Even a temporary change of the temperature should be avoided because of irreversible changes of the equilibrium of carbonic acid with erythrocytes and plasma proteins.

The determination is done exactly at the patient's temperature, no anticoagulant is added, contact with air is avoided, and the pH is read within a few seconds. However, we cannot measure at the patient's pressure and, what is worse, we do not know the influence of the blood pressure on the pH.

As changes in the blood pH occur very rapidly, the patient must be in a steady state for at least 10 minutes before the determination. All measurements should be avoided after alveolar rinsing in tracheotomized patients, after a heavy cough, vigorous movements, or any strong excitement.

With forced respiration the pH of a normal individual reaches values of 7.56 within 2 minutes. On holding the breath the pH falls to a value slightly below normal.

The capillary electrode developed by us has some particularities (Fig. 8). The very difficult problem of shielding against statical charges has been completely solved by letting the thermostat water circulate around all parts susceptible to these charges. For this reason the circulating water must not be distilled water but must contain some electrolytes. We simply use tap water.

Both ends of the glass capillary emerging from the electrode body are of polyethylene and, therefore, unbreakable. All creeping of the solutions and the development of creeping currents, especially on the upper part, are thus avoided. The upper part is covered by a glass cup with two lateral openings and a platinum wire entering the upper part of the capillary serving for the determination of the red ox potential.

The lower of the lateral openings is connected via a bottle to an aspirating aquarium pump. The capillary is filled by suction as soon as the other lateral opening of the cup is closed. A saturated calomel electrode is used as reference electrode. The temperature of the circulating water is adjusted to  $0.1^{\circ}\text{C}$ . to the patient's temperature.

As a measuring instrument we use an electronic compensator with an input resistance high enough to work with electrodes of 500 megohm resistance and an absolute accuracy of 0.01 pH unit.

Figure 9 shows the whole measuring unit mounted on a rolling table, Figure 10 the detail of the measuring chain.

After warming up the unit for 10 minutes, we calibrate the instrument with two standard buffers (phosphate buffer pH 6.81 and acetate buffer pH 4.65 at  $37^{\circ}\text{C}$ .). The measurement is usually made on capillary blood and immediately afterward the instrument is checked with the phosphate buffer, and should not vary more than 0.01 pH.

When the determination is finished the electrode is washed with water and then filled with a freshly prepared pepsine solution in 0.1N HCl. This is very important. Before we introduced the protein digestion with pepsine we never had reliable results. It is obvious that every unit of the apparatus and the rolling table must be grounded.

The way of collecting the cutaneous blood is very important. Patients with cold hands are given a warm hand bath for 20 minutes before collecting the blood. The finger, heel, or lobe of the ear is carefully cleaned with alcohol and water, dried, and a rather deep incision is made with a sharp lancet. The first drop of blood is discarded and the second drop is made as large as possible. Instantly the blood is drawn from the base of the drop, near or in the wound, into the capillary electrode avoiding air bubbles. As the volume of the capillary is about  $10\text{ }\mu\text{l}$ . most of the blood drop will remain in place. The measurement is made immediately and the electrode is instantly rinsed with water.

The same collecting technic is used for the  $\text{CO}_2$  determination. About 100

$\mu$ l. are collected with a polyethylene pipet and immediately delivered under paraffin.

The use of this unit is simple and the *pH* measurement can be done by technicians, doctors, or even nurses, after careful instruction. If the calibration with the two standard buffers is correctly done, the measurement of the blood *pH* will not present any difficulty, the electrode being nearly unbreakable. But as soon as one of the standard buffer readings does not correspond within 0.02 *pH* units to the theoretical value, very strict orders are given to instantly inform the specialist of the laboratory.

The main source of disturbances are the two seals of the glass capillary. As the electrical resistance at this point must not fall below 4000 megohm and as the temperature constantly oscillates between room temperature and 37° C., the sealing material must fulfill very high requirements. Any failure at this point is instantly recognized by a diminution of the potential difference between the two buffer values. Our last model has been working now without any trouble for 9 months, and is used nearly every day.

The measurement of the blood *pH* has become in the last year one of the most important determinations in the different clinics of the university hospital in Geneva. It is used in all diseases with respiratory involvement such as infantile paralysis, in all cases of artificial respiration, emphysema, cardiac insufficiency, barbiturate or salicylate poisoning, abdominal and lung operations (especially during curarisation), lung carcinoma, skull fractures, hormonal disturbances affecting the electrolyte balance, metabolic disorders due to renal insufficiency, and in ophthalmology. In addition to the *pH*, the total  $\text{CO}_2$  content of the serum is generally determined and the free carbonic acid, *p*  $\text{CO}_2$ , is calculated from these two values according to Henderson and Hasselbach.

The total  $\text{CO}_2$  and the oxygen saturation are determined with the micro-gasometer of Natelson, which gives us excellent results.

It is impossible to discuss here the role of the laboratory in respiratory or metabolic acid-base disturbances. One point however remains to be stressed, namely the relationship of arterial, venous and capillary *pH* values. Our normal values for capillary blood *pH* range from 7.37 to 7.41. The arterial value is on the average 0.01 *pH* units higher, and the venous on the average 0.02 *pH* units lower. However in pathologic cases the capillary blood closely approaches the arterial values in respect to *pH* and  $\text{CO}_2$ . This can especially be seen in following dramatic evolutions like alveolar or bronchial obstruction, atelectasis, or failure of the respiration apparatus, where the situation changes within seconds or minutes, while the venous blood changes much more slowly especially in respect to  $\text{CO}_2$ . Also in patients with chronic diseases such as emphysema or cardiac failure we observe that in capillary blood the respiratory situation is clearly represented. This is in accordance with the findings of Singer and his co-workers (2, 3).

### CONCLUSION

In conclusion we present new equipment which permits easy and accurate work on the ultramicro scale. We think that all clinical determinations can be made on this scale using 5, 10, 20, and, in rare cases, 50  $\mu$ l. of the sample.

The following determinations are done on ultramicro scale in our laboratory in serum, plasma, or whole blood: Chloride, total iodine and PBI, calcium, potassium, sodium, nonprotein nitrogen (hypobromite titration), total nitrogen (Kjeldahl and colorimetric), pH, total CO<sub>2</sub>, oxygen saturation, hemoglobin, proteins, fibrinogen; and in urine: pH, titratable acidity, and ammonia. Magnesium, sulfate, phosphorus, and glucose are in preparation and other procedures will follow.

We think that for inexperienced workers these methods are easier to learn and to execute than ordinary ones. For this reason they seem well suited for underdeveloped countries. A fully equipped laboratory can be installed in a small room, or even in a car.

As the reagent bottles hold enough liquid for 1000 to 5000 analyses the solutions very rarely need to be renewed if they are stable. Errors due to differences in reagents are nearly eliminated. Washing of glassware is reduced to a minimum, thus eliminating another important source of errors.

The fact that all the equipment is very sturdy and nearly unbreakable is another advantage for the user, less for the dealer. Nevertheless, all the equipment may soon be found on the market.<sup>2</sup>

### REFERENCES

1. Grunbaum, B. W., Schaffer, F. L., and Kirk, P. L., *Analyt. Chem.* **24**, 1487 (1952).
2. Singer, R. B., and Hastings, *Medicine* **27**, 223 (1948).
3. Singer, R. B., *Medicine* **33**, 1 (1955).

<sup>2</sup>Made by Beckman, Spinco Division, Palo Alto, Calif.

# Standardization in Clinical Chemistry

*Marcel H. Guillot*

IT WOULD BE USEFUL FIRST OF ALL to attempt to provide an adequate definition of what is to be called a true clinical method in biochemistry. It appears at once that not one but four different types of methods exist.

The first group includes elaborate analytic techniques and is intended to determine specifically one well-defined and pure chemical substance of physiologic or pathologic interest. A good example is the determination of uric acid in urine by the Ronchese method, based on the isolation of ammonium urate followed by the determination of uric acid with an iodine solution. This is a true analytic method.

The second group includes semispecific techniques which give only an approximate idea of the amount of substance in the medium. These techniques are often based on oxidation-reduction reactions and would become specific only after isolation of the compound to be determined; example: determination of pyruvic acid in blood.

The third group includes analytic techniques concerning a group of organic compounds somewhat similar in their chemical behavior but often not in biologic or pathologic importance. This is the case of the 17-ketosteroids.

Finally, a fourth group includes what we usually call biologic tests. These are techniques giving numerical data about some physical, chemical, or bacteriologic property, notwithstanding the lack of direct known relationship between these data and any well-defined qualitative or quantitative analytic characteristics of the sample. A typical example is Quick time in the study of blood coagulation.

## GENERAL DEFINITION

Thus, if clinical methods differ in such a way in nature, how is one to give a general definition of them?

One answer may be as follows: a clinical method is a method which has two main qualities required in routine work:

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1. It needs only a very small sample.
2. It can be run in a short time.

The result of the wonderful expansion of the research work in biochemistry has been a general tendency to increase the number of biochemical components to be determined on one hand and the number of different methods available for the determination of each of them on the other hand. It will obviously become more and more important in the future to appraise critically in each case, the technic to be used even in daily routine clinical work. We shall have to pay attention to a number of points:

1. The need for national and, if possible, international standardization of methods
2. The need to limit the number of methods to be used in routine clinical work (the number of methods available for research work being, of course, unlimited)
3. The need to use the above-mentioned classification as a reference when writing a scientific paper about any method

It would be expected, for instance, that the exact concentration of a biochemical constituent would be indicated only in the case of a true analytic method of determination, whereas only the approximate amount of a substance defined by its name would be given when dealing with a semispecific method. In the case of a method referring to a group of substances, only estimates would be reported, whereas in the case of biologic tests, it would probably be best to mention the rough data obtained in well-defined conditions without mentioning any component by name.

Of course, in any of the four cases the technic used should be explicitly mentioned and should be, if possible, a well-known and standardized one.

### LONGEVITY

Attention should now be drawn to another point. Most physicians feel strongly that the accuracy of a clinical routine method does not matter much. What is most important in their opinion is that the life expectancy of the method is long and the method stay without any modification. The most important biologic methods related to the clinical work done in the past were very often not truly analytic, and research workers may have looked at them with disapproval, but they were useful tools in the hands of physicians who used them year after year and were able to gather at last on one single chart the data they had accumulated during the past 10 or 20 years. Recent scientific developments may have had very bad consequences in this field by shortening the life of all methods more and more; even if the new methods prove to be better than the old ones, they might happen to be of relatively little value in clinical research if physicians do not have the opportunity to make a long and quiet use of them.

It seems, therefore, that standardization is good at any time but it ought to



be done carefully in order to keep each standardized method in its unchanged form for as long a time as possible. In this connection, it might be useful to quote the French President of the Society of Hematology, Prof. Paul Chevalier, saying that he did not want to know if the Gowers-Sahli method for hemoglobin determination in blood was good or bad for he had based his entire classification of anemias on it and he did not easily accept the suggestion of letting everything become obsolete.

Of course it would be preposterous to put an end to such evolution since it could mean the end of any research or scientific progress. Every day each of us receives pressing suggestions from other physicians to try and find new methods for determination of the various biochemical constituents of the blood. Furthermore, in our conversations with those doing pure clinical work, we often feel that our analytic sciences are unfortunately poor and deficient, due to the fact that we are unable to submit a medical hypothesis to the required control of experimental laboratory research.

We may thus be asked to simplify and standardize and at the same time to expand possibilities of the technics and to diversify them. An equilibrium should be found, and unfortunately it will prove to be more and more difficult to reach.

#### ACTIVITY IN FRANCE

As it has just been done by Wootton for England, an attempt will now be made to give you a rapid view of what was done hitherto in France.

Until World War II there was only one association interested in biochemistry in France. Its name was *Société Française de Chimie Biologique* (French Society for Biologic Chemistry). But during the war, in 1942, it appeared necessary to set up a new organization devoted only to clinical work. Since many scientists in clinical work in France were experienced in various fields of biology, the chosen name was *Société Française de Biologie Clinique* (French Society for Clinical Biology).

Soon after, as a consequence of increasing applications of biology in medicine, a third French organization was created, the *Société de Biologie Médicale* (Society for Medical Biology). In spite of the similarity of their names, they agreed on a tentative program of different activities. The new Society for Medical Biology was established mainly for papers on biologic research of interest in human pathology or physiology, whereas the Society for Clinical Biology would mainly publish studies on biologic technics and their development in relation to clinical needs. As for the elder Society for biologic chemistry, this organization would mainly publish as it had always done, papers on general plant and animal biochemistry. Things being as they were in France, the Society for Clinical Biology had to deal with any problems concerning methods, their standardization and improvements, etc.

The main result achieved so far has been the permanent organization of a



system of annual meetings which takes place each year in Paris. This time of year was chosen because the French Ministry of Health requires the staff members of clinical laboratories all over France to come to Paris in the middle of November to attend an advanced training course at the official *École de la Santé*, which is a government institution apart from the university where lectures are delivered during one week on a number of important new topics related to laboratory work. The following week, the Society for Medical Biology holds a 2- or 3-day session where reports and short papers are presented. At the end of the meeting, a symposium takes place dealing only with technical matters.

We thought it more advisable to focus the interest this year on only one very-well-defined subject. For instance, at the meeting last November we were only concerned with electrophoresis in clinical biology and, in particular, paper electrophoresis.

The reports given at this symposium were limited to a review of all the technical aspects of importance, such as:

Conditions required for a good result to be obtained from a physical standpoint;

Comparison between Tiselius cell electrophoresis and paper electrophoresis made on the same serum samples by two independent workers in various pH and buffer conditions;

Dyeing of protein spots on the paper;

Computation technics to evaluate areas of the curves in order to provide data about the amount of each protein;

What can be expected at the present time of the methods and the best way to use it in clinical routine work.

The lecturers have been asked to meet several times during the previous year to agree on what they were to discuss so that they knew exactly on what part of the whole subject they would have to speak. Then at the end of the symposium a general discussion would give every member of the meeting an opportunity to obtain all the information he wanted.

Our purpose is not at all to try to promote new research work but only to help clinical laboratory directors in their difficult daily tasks. The next meeting will be concerned with cholesterol determination in the serum by the classic Liebermann method. A team of five scientists has been organized and has been working for several months in Paris on a comparison of various technics in various conditions on the same serum samples with a standard control method as the reference. In this way we hope to be able to give French biologists a reliable opinion on the possible origin of discrepancies among the data published every day in clinical papers. We also hope that this work will make biochemists more aware of the importance of a general agreement on technics to be used.

If such work could be accomplished in several countries at the same time, the

international standardization of methods would become easier. It might be possible to gather three or five individuals who have enough experience to decide what is useful and what is possible from the point of view of standardization in their country and within the limits of a specialized field. They would find out, when the time of international standardization would come, that many dangerous illusions should be avoided and that time and effort should not be wasted in suggesting unrealistic programs.

# Standardization of Methods in Clinical Chemistry

David Seligson

THERE IS A CONSTANT STRIVING for better analytical procedures, for elimination of errors, and for increased efficiency without loss of accuracy in clinical chemistry. Apparently, we do not want to run the old methods. We are seeking better methods and higher standards of performance, for we all know how much room exists for reasonable improvement.

We need exact data to practice good medicine. Bad data is often worse than no data to a physician or investigator. The statement, "it is good enough for clinical use" is a rationalization, which chemists should not tolerate. I have seen laboratories provide results on serum chloride, for example, which were in error by 3 times the normal physiologic variation. I should like to see our methods give results with errors of one-third or less the physiologic range. We can define the error of a method and we should run the method within this error. We need specific and accurate methods to determine physiologic variations, free of methodologic variations.

We need methods which can be easily and accurately standardized, and where standardization is difficult, we need good secondary standards which can be made available to all chemists. We also need good methods in order to analyze accurately and evaluate the new "control" or "standard" serums which are now appearing on the market.

With specific and accurate methods we shall have uniform biologic data, the world over. We are entering an era in which we should discard the phrase "normal value for this hospital," which implies that either patients or methods vary widely from hospital to hospital, and which, therefore, precludes interchange of data between these hospitals.

These methods should also be economical to perform. I feel strongly that a well-chosen method often can give less error at less expense than many popular methods. For example, we use an electrometric micromethod for chloride

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The author is grateful to Miss Jean Marino for use of some of her data and help in preparation of this report. Some of the work mentioned has been sponsored by the Becton, Dickinson Company.

which is run directly on serum and which has an error of less than 1 per cent. A serum sample can be analyzed and reported within 2 minutes. Compare this to the old Volhard modifications. The cost of medical care is rising, partly because of laboratory costs and the increased need for laboratory data in the practice of medicine. We should consider costs in our development of better methods. However, we should not compromise quality for cost.

### STANDARDIZATION

Now, concerning standardization, I should like to define standardization as I am going to use the word. In analytical chemistry we standardize an alkali against a weighed primary acid standard. We often use this standard alkali to standardize another solution of acid. In this case the standard alkali is a secondary standard. In clinical chemistry, in general, we compare the color obtained from a sample after a series of reactions against the color of a primary standard. Many of us in this country refer to this procedure as standardization. It is in this sense that I wish to refer to standardization of methods. An example of a secondary standard is a stable serum carefully analyzed for protein nitrogen by a good Kjeldahl procedure and used for standardizing a biuret method.

In traditional analytical procedures the desired constituent is isolated and weighed or titrated. In clinical chemistry the samples we use often contain the desired constituent in minute amounts. For example, we are often fortunate in having for analysis a sample containing a substance present to the extent of 0.1 per cent, as glucose in blood. We resort to photometry for the large majority of final measurements because it is a sensitive procedure and reasonably simple, and instrumental errors can be kept within 1 to 2 per cent. We use the photometer as a device for comparing the color of the reaction mixture containing the sample with that of our primary standard. In a few methods where a carefully selected spectrophotometer of high precision is used, the known extinction coefficient for a particular substance may be used for measuring such compounds as oxyhemoglobin or cyanmethemoglobin. The large majority of compounds measured, unfortunately, are not uniformly chromogenic from day to day. Therefore, the photometric instrument cannot be used for direct measurement, but only for comparison against standards run concurrently.

A few assumptions are made when we compare our unknown sample to our known primary standard. First, we assume that color does not appear from other substances in the sample. Second, that catalysts present in the sample do not alter the reaction in any way. Third, that samples compared to standards run concurrently through the entire analytical procedure will lead to the correct result. Fourth, that substances present do not alter the purity or amount of color formed.

### STANDARDIZATION OF BILIRUBIN

I wish to discuss standardization of the bilirubin method in order to demonstrate how some of these assumptions are invalid and I shall take the widely used method of Malloy and Evelyn (1) as modified by Ducci and Watson (2) as an example. I shall discuss only total bilirubin. In this method diluted serum is treated with an acid diazo reagent and alcohol and then measured in a photometer. The reading is compared with that of a bilirubin standard, usually made in chloroform and methanol and treated with acid diazo reagent. In the sample we have serum proteins and the added acid while in the standard we have no proteins. It is apparent that the reaction mixture containing the sample will have a higher  $pH$  as a result of the serum and its proteins, while the standard free of these constituents will have a slightly lower  $pH$  and will contain no proteins. Let us look at the effect of these two factors,  $pH$  and proteins, on the serum bilirubin determination.

Figure 1 shows the effect of  $pH$  of the final reaction mixture on the wavelength at which maximum absorption of bilirubin after diazotization occurs and the effect on the optical density at peak absorption. It is interesting to note that the peak absorption of reaction mixtures involving pure bilirubin samples occurs at  $560\ m\mu$  and that maximum optical density is obtained at  $pH\ 1.9$ , whereas considerably less density is obtained at lower and higher  $pH$ . When a serum was run in a similar fashion, the peak absorption was  $535\ m\mu$  instead of  $560$ , and the  $pH$  effect on optical density was marked.

Figure 2 demonstrates that when serum albumin is varied but  $pH$  maintained constant, different densities are obtained. The maximum density was observed at 5.0 Gm. per 100 ml. of albumin in the serum sample. Note that when albumin is 1.25 per cent of the serum, as is often the case in severe liver disease, the density resulting from bilirubin is considerably less than that observed at the 5 per cent level. Also, larger amounts of albumin reduce the optical density and tend to shift the peak of absorption to a slightly lower value.

In order to standardize this method and take into account some of these factors by controlling  $pH$  and assuring the presence of serum proteins, we obtain a serum low in bilirubin and measure it. Then we repeat the analysis on this serum but with an added known amount of bilirubin. For example, we place 3.0 ml. of a 1:6 dilution of serum into two 18-mm. test tube cuvetts. We add 1.0 ml. of acid to one tube, 1.0 ml. of acid diazo reagent to the other, follow with 4.0 ml. of methanol to each tube, and read. For the standard we add 4.0 ml. of methanol containing  $20\ \mu g.$  of bilirubin to the diluted serum acid diazo mixture and diluted serum acid mixture. The reaction mixture so prepared is equivalent to a hypothetical serum containing 4.0 mg. per 100 ml. more bilirubin than it previously did. Since the color follows Beer's law up to this amount, we calculate a constant factor from this 4.0 mg. per 100 ml. and the optical density obtained with serum and serum plus bilirubin.

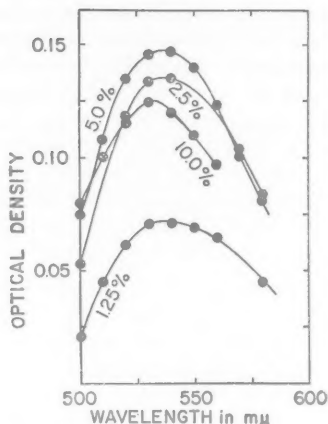
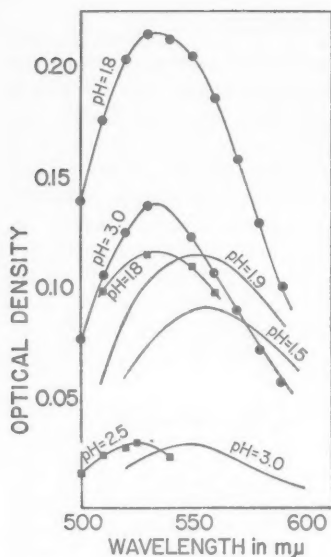


Fig. 1. Effect of pH of final reaction mixture on absorption maximum and peak absorption of diazotized bilirubin. The curves free of symbols represent bilirubin standards containing 3.2 mg. of bilirubin per 100 ml. of chloroform-methanol solution. The curves with squares are from serum containing 2.9 mg. of bilirubin per 100 ml. The curves with dots are from serum containing 5.0 mg. of bilirubin per 100 ml. Fig. 2. The effect of serum albumin concentration in the sample on absorption maximum of diazotized bilirubin. Each curve is labeled for the sample albumin concentration.

This, then, is a means of standardizing the method for serum bilirubin. The pH is controlled better by assuring the presence of serum. This also assures a better control of the albumin effect; but not complete control. We have found this type of procedure valuable in other standardizations.

I should like to go to the bromsulfalein method as another example in which some of these assumptions are not completely valid. Here, one adds alkali to serum containing bromsulfalein and reads the photometer. If one wishes to avoid the interference from turbidity, hemolysis, or jaundice, one reads at 2 or 3 wavelengths in order to make corrections. We observed visually that bromsulfalein in serum had a different color from the equivalent amount in water. On studying this we observed that BSP in water absorbed maximally at 580  $m\mu$ , as shown in Fig. 3. When the equivalent amount of BSP in serum was studied, the peak absorption occurred at 592. Furthermore, in the presence of serum proteins the optical density had diminished. We also noted that increasing albumin decreased the optical density, as shown in Fig. 4. Since we

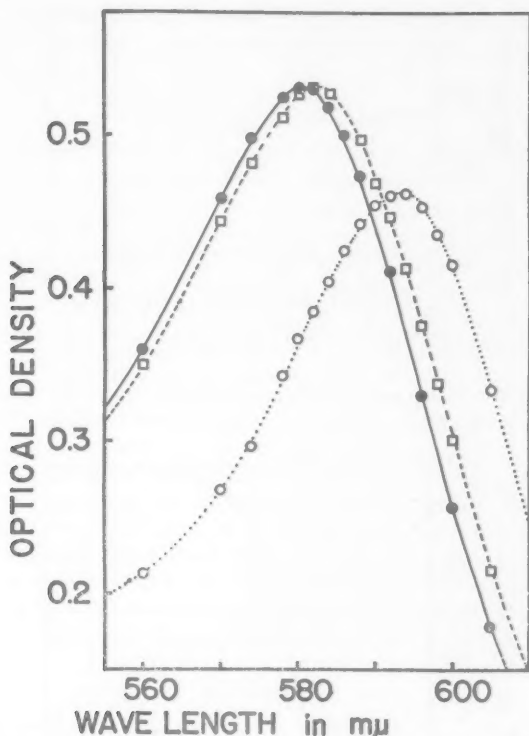


Fig. 3. Spectral-absorbance curve of bromsulfalein at pH 10.3 with and without albumin. Curves with dark circles are BSP. Curves with squares are BSP, albumin, and *p*-toluenesulfonate. Curves with light circles are BSP and albumin.

considered this effect to be due to protein binding, we added a large excess of *p*-toluenesulfonate to our alkaline buffer to reduce this effect. The BSP-serum curve then almost exactly resembled that of BSP in water, as shown in Fig. 3. This then eliminated the albumin effect.

Figure 5 shows a dissociation curve of BSP. Here 98 per cent of BSP color is obtained between pH 7.3 and 10.3. We observed that by confining our photometric readings to these two pH's we could eliminate interferences from bilirubin up to 35 mg. per 100 ml., from hemoglobin up to 180 mg. per 100 ml., and from grossly visible turbidity. Furthermore, we found it unnecessary to take readings at more than one wavelength under the circumstances.

The method is performed as follows: Into an 18-mm. test tube cuvet are placed 0.5 ml. of serum and 3.5 ml. of alkaline buffer containing *p*-toluene-

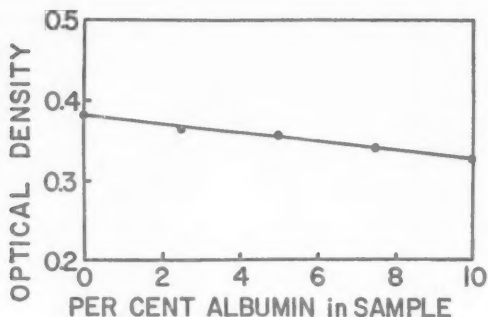


Fig. 4. Effect of albumin on optical density of bromsulfalein in serum. The abscissa represents Gm. per 100 ml. of an albumin solution containing BSP (25 per cent retention), which was run as a hypothetical serum.

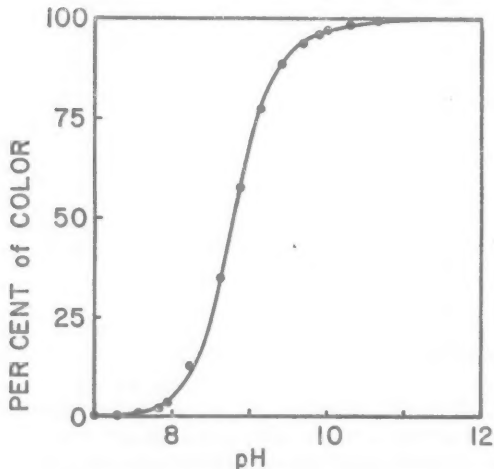


Fig. 5. Dissociation curve of bromsulfalein.

sulfonate, which brings the final reaction mixture to pH 10.3. We read at 580  $m\mu$  and then add 0.1 ml. of acid phosphate, which brings the pH to 7.0, and we read again. The optical density difference is a measure of the bromsulfalein. The method is easy to perform and gives accurate data even when marked hemolysis, jaundice, or turbidity are present. In this case we can use pure BSP solutions for standardization without concern for the above interferences or concern for serum albumin.



It is my impression that BSP retention values up to 10 per cent for healthy persons as has been recorded in the literature reflect combined physiologic and analytical variations.

### SUMMARY AND CONCLUSION

Concerning our problem of improving quality of performance of clinical chemistry, we have several factors to consider. The one I have discussed is standardization. Running concurrent standards will be of great help. I want to emphasize that certain methods, as I have shown, need control of some of the conditions of the reaction in order to make the chemical substance being measured react the same way in sample and in standard solution. The use of a standard serum as a secondary standard may be of great help. One of our problems is to see how these can be certified and to determine their stability for the desired constituents.

I should like to mention for strong condemnation the use of precalibrated photometers to replace standardization. I think the instrument houses which foster this policy in their advertising do our field a disservice.

We should maintain a high quality of accuracy by running concurrent primary or secondary standards with our samples, and by improving the methods which we use. If we do not, we shall find that incompetent persons will invade our field and provide inaccurate data in a competitive manner. Modern medicine is improving our lot as chemists and emphasizing our important role. We have to meet our responsibilities by providing work of high quality and by leading the field of clinical chemistry.

### REFERENCES

1. Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.* **119**, 480 (1937).
2. Ducci, H., and Watson, C. J., *J. Lab. Clin. Med.* **30**, 293 (1945).

# Experiments of Chronic Diabetic Symptoms Caused by Xanthurenic Acid, an Abnormal Metabolite of Tryptophan

Yahito Kotake

SINCE 1950 WE HAVE CONDUCTED a series of experiments on the diabetic symptoms in albino rats caused by the administration of xanthurenic acid (XA), an abnormal metabolite of tryptophan. Our studies have come to present some conclusive evidences that XA is related to the development of human diabetes which has been generally considered attributable to an inadequate food intake and also such evidence suggest a close relation between the animal and human diabetes.

Our experiments have confirmed XA to be a substance produced in the body as result of taking too much sodium salt of fatty acid (representing fat) and tryptophan (representing animal protein). Our experimental method may be a new road to the study on human diabetes.

## 1. TRYPTOPHAN METABOLISM

Since our studies are closely concerned with the tryptophan metabolism, a brief explanation will be given on it. The normal metabolism of tryptophan has so far been ascertained by Yashiro Kotake to be the following: tryptophan is first converted through kynurenine into anthranilic acid and finally into 5-hydroxy-anthranilic acid. He has confirmed that the enzymes involved in these chain reactions are tryptophanpyrrolase and kynureninase, and that 5-hydroxy-anthranilic acid thus produced has several important physiological actions. Another metabolic path as proposed by Beadle *et al.* is as follows: tryptophan is first converted through kynurenine into 3-hydroxyanthranilic acid and finally into nicotinic acid. Braunstein has made it clear that kynureninase keeps pyridoxal phosphate as co-enzyme. It follows, therefore, that, in albino rats in vitamin B<sub>6</sub> deficiency, these two series of reactions are in-

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hibited, so that XA is produced from tryptophan as found by Lepkovsky and excreted in the urine as an abnormal metabolite. XA was found for the first time by Musajo as one of the tryptophan metabolites.

## 2. ADMINISTRATION OF TRYPTOPHAN CONCURRENTLY WITH SODIUM SALT OF FATTY ACID AND URINARY EXCRETION OF XA

In the first series of experiments, we administered to albino rats simultaneously by oral route large amounts of both the sodium salt of fatty acid and tryptophan (for instance, 0.3 Gm. of sodium butyrate and 0.1 Gm. of tryptophan), and confirmed the excretion of a large amount of XA in the urine a few hours later. The same effect was observed not only by sodium butyrate alone but also by other fatty acid salts as shown in Table 1.

Table 1 clearly shows that, compared with the administration of tryptophan alone, a markedly increased excretion of XA was observed when any of the various fatty acids was administered together with it. We administered to albino rats 0.4 Gm. of sodium butyrate simultaneously with 0.1 Gm. of tryptophan, and succeeded in separating XA in crystalline form from their urines.

This experiment, with reference to that of Lepkovsky, leads us to a conclusion that the administration of large doses of the sodium salt of fatty acid to albino rats unfailingly results in the deficiency in vitamin B<sub>6</sub> or the inhibition of vitamin B<sub>6</sub> activity; namely, the inhibition of ATP→Pyridoxal-transphosphatase.

The above experiment proves that the excessive intake of fat greatly affects the normal tryptophan metabolism to finally lead to the formation of XA in the body.

## 3. DIABETOGENIC EFFECT OF XA ON ALBINO RATS

We first noted its close resemblance in its chemical structure to oxine which was discovered by K. Okamoto as one of the diabetogenic substances, and further observed that some of the albino rats manifested cataract when reared

**Table 1.** THE AMOUNT OF XA EXCRETED IN 24-HOUR URINE XA WAS DETERMINED BY GLAZER'S METHOD. TO ALBINO RATS, WEIGHING 120 GM., 0.1 GM. OF TRYPTOPHAN AND 0.4 GM. EACH OF SODIUM SALT OF VARIOUS FATTY ACIDS WERE SIMULTANEOUSLY ADMINISTERED

<i>Substance administered</i>	<i>Amount excreted</i>
	mg.
Tryptophan alone	1.61
Tryptophan plus sodium acetate	5.37
Tryptophan plus sodium propionate	8.79
Tryptophan plus sodium butyrate	9.89
Tryptophan plus sodium valerianate	9.64
Tryptophan plus sodium palmitate	9.61
Tryptophan plus sodium stearate	8.57
Tryptophan plus sodium oleate	10.49

for 150 days after several repeated administrations of sodium butyrate and tryptophan. These findings served as a clue to the belief that XA can be a factor causative of diabetic symptoms in rats.

We nextly investigated the effect of XA on the blood sugar level. We fed albino rats for two weeks on a synthetic diet consisting of (in per cent): casein 22, McCollum salt mixture 6, agar-agar 3, yeast 2, butter 10, sugar 5, and starch 52.

To one group of the animals, sodium butyrate and tryptophan were orally administered (Fig. 1) and, to the other group, an aqueous pH 7.4 solution of crystalline XA separated beforehand from the urine of albino rats by intraperitoneal route (Fig. 2). The variations in the blood sugar level was estimated by the Hagedorn-Jensen's method on each group. In all the 30 cases, a temporary hyperglycemia occurred an hour after treatment to be followed by an intermediary period of hypoglycemia and finally the continued hyperglycemia ensued, accompanied with glycosuria.

Taking into consideration the chronic nature of human diabetes, we then studied on the development of chronic diabetic symptoms by xanthurenic acid. As the first step of such study, we examined the blood sugar level and body weight after feeding a high fat and tryptophan diet. Albino rats were fed on a diet containing rich fat (butter 35%) and casein (25%) for 240 days, and the blood sugar level and body weight were plotted against time of feeding as given in Fig. 3. About 1 mg. per day of XA was excreted in the urine even in this case, since the diet consisted of large proportions of fat and casein. In order to promote the accumulation of XA in the body, 10 mg. of tryptophan were added daily to the said diet for the first 30 days of experiment.

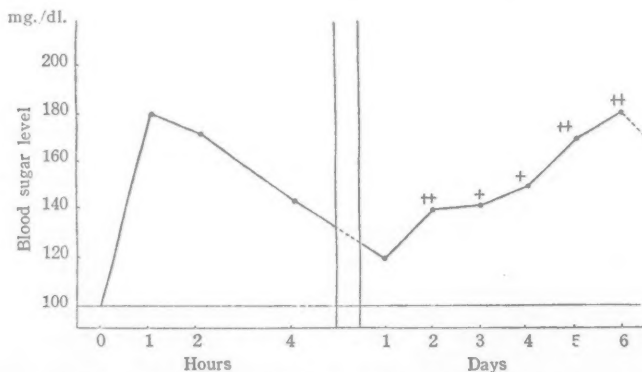


Fig. 1. Blood sugar curve. Male albino rats, weighing 150 Gm. were employed. 0.4 Gm. of tryptophan and 0.8 Gm. of sodium butyrate were orally administered. ++, + indicate the amount of glucose in the urine determined by Benedict's method.

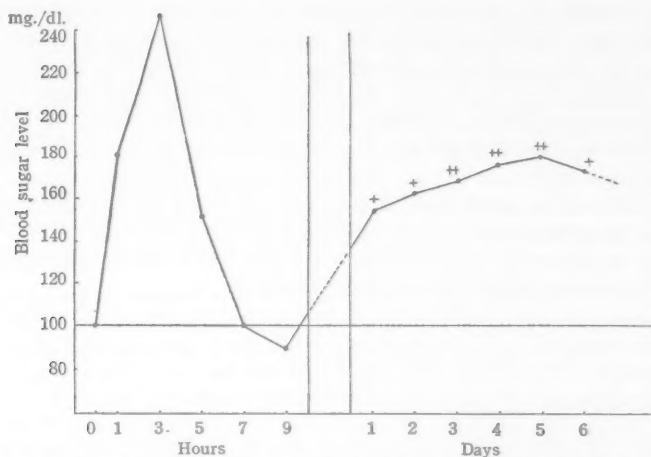


Fig. 2. Blood sugar level. Male albino rats, weighing 150 Gm. were employed. Xanthurenic acid 120mg./kg. was injected intraperitoneally. ++, + indicate the amount of glucose in the urine determined by Benedict's method.

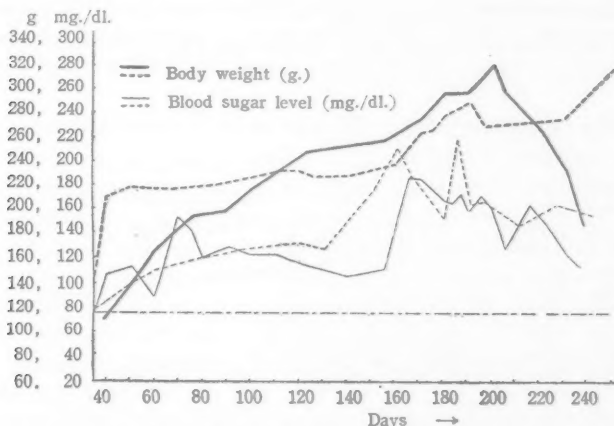


Fig. 3. Body weight and blood sugar level after administering high fat and casein diet. Albino rats were fed for a long period on a diet containing much fat and casein.

It resulted that 2 to 3 mg. of XA were found daily in the urine. In parallel with this, the albino rats started to gain the body weight steadily and regularly till they showed a particularly marked increase on about the one hundred seventieth day to attain the maximum of 325 Gm. with manifestation of obesity

due to excessive fat. The blood sugar level was also found to rise gradually till it reached the maximum of 170 to 200 mg. per 100 ml. on about the one hundred seventieth day, following the pattern of body weight. For the determination of the true blood sugar level, the King and Garner's method was adopted, using a sample of 0.02 ml. of blood. With this method, we invariably obtained a lower value by about 30 mg. per 100 ml., as compared with that of Hagedorn-Jensen's.

From the results stated above, it has become clearer than ever that there is a close interrelation between the administered diet and the development of diabetic symptoms in these animals, and the probable nutritional etiology of the disease seems to have become more convincing.

As the second step of this series of experiments, we then checked the blood sugar level and the body weight after feeding the albino rats on a vitamin B<sub>6</sub> deficient diet. We fed them over a period of 240 days on a vitamin B<sub>6</sub> deficient diet consisting of (in per cent): Hammarsten casein 20, McCollum salt mixture 4, salad oil 2, and sugar 74, containing in addition per gram of the diet the following vitamins (in gamma): Thiamine hydrochloride 3.3, choline chloride 166.0, inositol 333.0, p-aminobenzoic acid 200.0, calcium pantothenate 13.3, and riboflavin 6.6. Keeping the rats under such condition as an adequate excretion of XA can be maintained, the blood sugar level and body weight were plotted against the experimental days as shown in Fig. 4.

From the twentieth day, 10 mg. of tryptophan were added to the diet daily for 60 days in order to promote the production of XA in the body. The albino

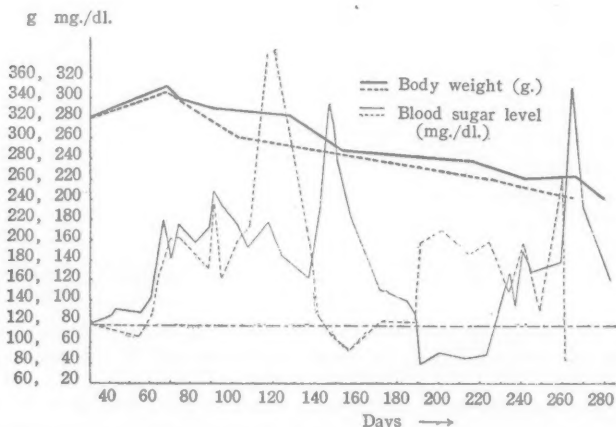


Fig. 4. Body weight and blood sugar level after feeding on a vitamin B<sub>6</sub> deficient diet. Albino rats were fed for a long period on a diet deficient in vitamin B<sub>6</sub>.

rats, each weighing about 260 Gm. at the outset, showed a gradual weight gain for the first 30 days and then a loss to be followed by a marked drop on about the one hundredth day, and died on the two hundred thirtieth to two hundred fiftieth day. In the meantime, a rapid increase in the blood sugar level was noticed on about the thirty-fifth day, indicating a simultaneous vitamin B<sub>6</sub> deficiency, and the blood sugar level rose thereafter to 160 to 200 mg. per 100 ml. During the whole course of the experiment, the true blood sugar level of about 400 mg. per 100 ml. was observed in some of the cases. The urinary excretion of XA was recorded from about the thirtieth day, amounting to 4 to 7 mg. daily.

These findings may offer a conclusion that, unlike alloxan, XA has an accumulative effect on the development of diabetic symptoms. It is worthy of note, with consideration to the fact that human diabetes generally progresses chronically.

The histological findings are as follows: One group of albino rats were fed on a vitamin B<sub>6</sub> deficient casein diet and the other on a diet rich in fat and casein, respectively over a long period. They were sacrificed when they manifested hyperglycemia and the pancreas tissue was examined histologically after being stained by the Gomori's alum-hematoxylin-phloxin method.

A remarkable drop of stainability, a decrease in granula, many vacuole formations, and marked collapse of the protoplasm, etc. were noticeable in the  $\beta$ -cells of the Langerhan's islets.

#### 4. XANTHURENIC ACID IN THE URINE OF DIABETIC PATIENTS

For establishing the possible relation of human diabetes to the diabetic symptoms caused in albino rats by XA, 23 diabetic patients under treatment at the Hospital attached to the Wakayama Medical College were examined. One liter of urine was collected from each patient, filtered, and concentrated. The urine of normal healthy people was treated exactly in the same way as a control and each solution was subjected to paper chromatography, using a mixture of butanol, acetic acid and water (4:1:1) as a developing solvent.

The results are presented in Fig. 5. The color reactions and the R<sub>f</sub> value of each spot are shown in Table 2.

F<sub>4</sub> of column B undoubtedly corresponds to XA of column C on the basis of color reaction and R<sub>f</sub> value, and was identified as XA. In the same way, F<sub>3</sub> is considered to indicate kynurenine. The urines of all the 23 patients were proved to develop these chromatographical findings without a single exception.

That XA in a free form was invariably present in the urines of diabetic patients—the fact leads us to conclude that there is an undeniable etiological relation between free XA and human diabetes.

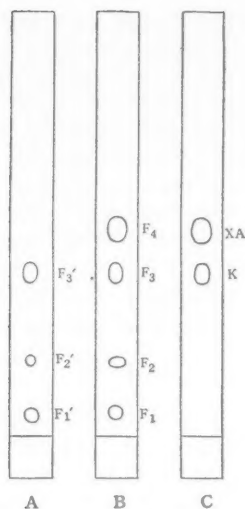


Fig. 5. XA, xanthurenic acid; K, kynurenine; A, healthy urine; B, patient urine; C, control.

Table 2. SPOTS DETECTABLE BY PAPER CHROMATOGRAPHY

	A			B			C	
	F1'	F2'	F3'	F1	F2	F4	K	XA
Rf value	0.06		0.40	0.06	0.41	0.50	0.41	0.50
Bratton-Marshall	—		+	—	+	—	+	—
Ehrlich	+		—	+	—	+	—	+
Pauly	+		—	+	—	+	—	+
Millon	—		—	—	—	+	—	+
Fe+++	—		—	—	—	+	—	+
Ninhydrin	+		+	+	+	—	+	—

A, the urine of normal healthy subject.

B, the urine of diabetic patient.

C, mixed solution of kynurenine (K), and XA.

## 5. THE INHIBITIVE ACTION OF 5-OH-ANTHRANILIC ACID ON THE FORMATION OF XA

We conducted experiments for investigation of the inhibitive action of 5-hydroxy-anthranilic acid on the formation of XA and the subsequent development by the latter of diabetic symptoms in albino rats administered sodium butyrate and tryptophan.

A group of rats given sodium butyrate and tryptophan orally were set



aside as the control, while the rest further given 0.2 mg. per kilo of 5-hydroxy-anthranilic acid twice were set aside as the test animals.

Table 3 shows a markedly decreased urinary excretion of XA in the test group. It is certain, therefore, that 5-hydroxy-anthranilic acid can markedly diminish the formation and excretion of XA in the body.

In this case, no continued hyperglycemia was detected, either.

#### 6. ACTION OF ETHEREAL SULFATE OF XA AS THE INHIBITING AGENT AGAINST THE DIABETOGENIC PROPERTY OF XA

We isolated 4-8-diethereal sulfate of XA from the urine of albino rats administered XA and conducted with it the following experiments.

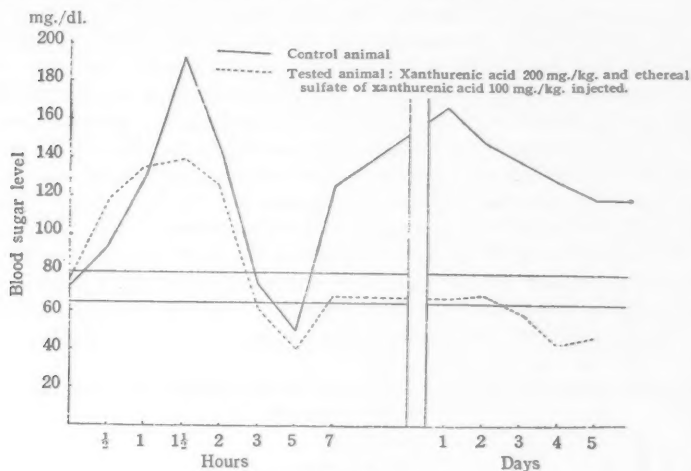


Fig. 6.

Table 3. XANTHURENIC ACID EXCRETION

Experimental rat No.	Control mg.	Test mg.
1		2.1
2	9.8	1.4
3	8.24	1.85
4	7.52	2.38
5	8.19	3.6
6	7.73	1.8
7		1.22
8		2.5
9		2.4
10		1.52
Average	8.29	2.07

A control group was injected 200 mg. per kilo of XA and a test group was similarly injected 100 mg. or 75 mg. per kilo of ethereal sulfate of XA and 10 minutes later re-injected intraperitoneally 200 mg. per kilo of XA.

In Figure 6 are recorded the variations in the blood sugar level of each group examined in terms of time. In each of the test animals, the same initial hyperglycemia as that in the controls was noted in a slight degree, but the continued hyperglycemia that was expected to appear 24-hours later was completely inhibited, and none of the diabetogenic symptoms developed.

## 7. EFFECT OF XA ON CARBOHYDRATE METABOLISM

### A. EFFECT OF XA ON PHOSPHORUS METABOLISM IN VIVO

It has been established from the above experiments that the XA is the diabetogenic substance, and we conducted an attempt to investigate the effect of XA on the glycometabolism as the first step of research for the cause of its diabetogenicity. We first examined the phosphorylation of carbohydrate which, among various steps of glycometabolism, occupies an important part.

It seems interesting in this connection to investigate the effect of XA on the phosphorus metabolism with relation to the diabetic symptoms in rats.

A number of rats were injected an aqueous pH 7.2 synthetic XA intraperitoneally in the dosage of 300 mg. per kilo of body weight. The blood sugar was determined by the King-Garner's method, the blood phosphocreatine and inorganic phosphate by the King and Shimazono-Takahashi's method, the urinary XA by the Glazer and Mueller's method, and the urinary total creatinine by the Folin and Wu's method. The results are illustrated in Figs. 7 and 8.

It will be learned from Fig. 7 that the inorganic phosphate in the blood and

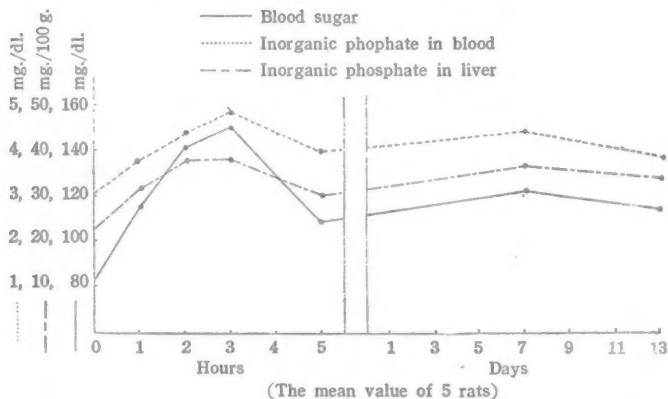
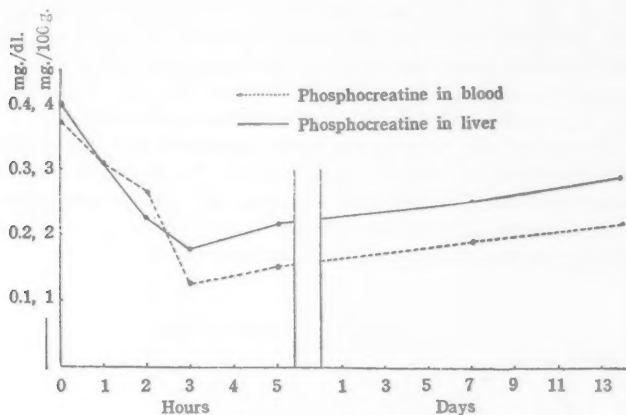


Fig. 7.

liver showed a distinct increase to reach the maximum 2 to 3 hours after the injection. On the contrary, the phosphocreatine in the blood and liver showed a decrease (see Fig. 8).

#### B. EFFECT OF ADMINISTRATION OF GLUCOSE

It was examined in the following way: When the animal showed a hyperglycemia due to the injection of XA, to high fat food or to lack of vitamin B<sub>6</sub>, 2 grams per kilo of glucose were given to the animal orally as a 50% aqueous solution, and the blood inorganic phosphate was determined. The results are shown in Fig. 9.



(The mean value of 5 rats)

Fig. 8.

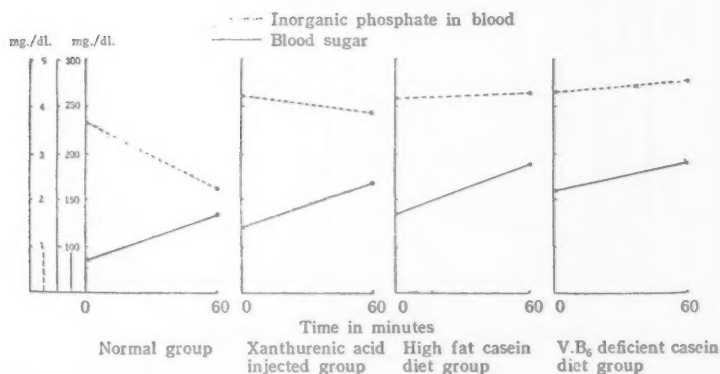


Fig. 9.

As indicated in Fig. 9, the blood inorganic phosphate in the healthy controls decreased. This means that phosphorylation of sugar went on smoothly. But this was not the case with the animals made diabetic by the said procedure. Here, the decrease in inorganic phosphate did not occur notwithstanding the increase in the blood sugar in diabetic patients (see Fig. 10).

### C. EXPERIMENT ON HEXOKINASE

In further pursuit of this line, both yeast and animal hexokinases were prepared. The activity of these two hexokinases was examined by measuring the liberated  $\text{CO}_2$  with the aid of Warburg's manometer.

Hexokinase thus prepared was dissolved in substrate glucose in such a concentration that there could be no effect of the presence of myokinase, and thus the resultant enzyme system was examined for its enzyme activity.

The results recorded in Figs. 11 and 12 indicate that XA in the final  $10^{-3}$  concentration proved highly inhibitive against hexokinase activity.

It is evident from the experiments herein presented that XA inhibits the hexose phosphorylation *in vivo* as well as *in vitro*. The finding further leads to the consideration that XA which thus inhibits the glucose metabolism must help store a large amount of glucose into the blood; thereby giving rise to such diabetogenic symptoms as hyperglycemia and glucosuria.

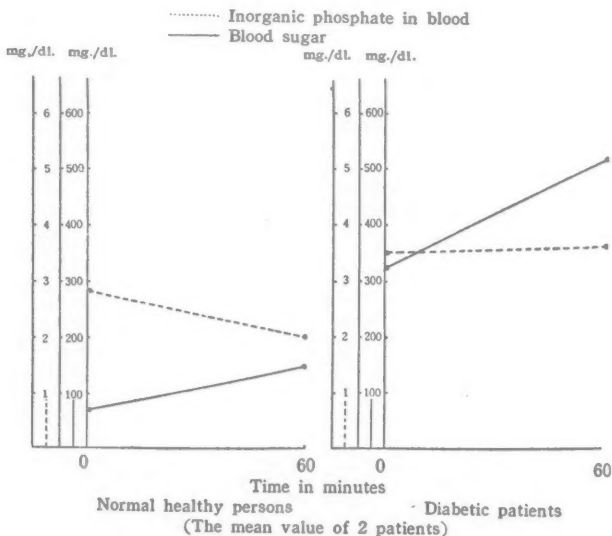


Fig. 10.

Yeast hexokinase (prepared by L. Berger's method)

1. in final xanthurenic acid  $10^{-6}$ M
2. control
3.  $10^{-5}$ M
4.  $10^{-4}$ M
5.  $10^{-3}$ M

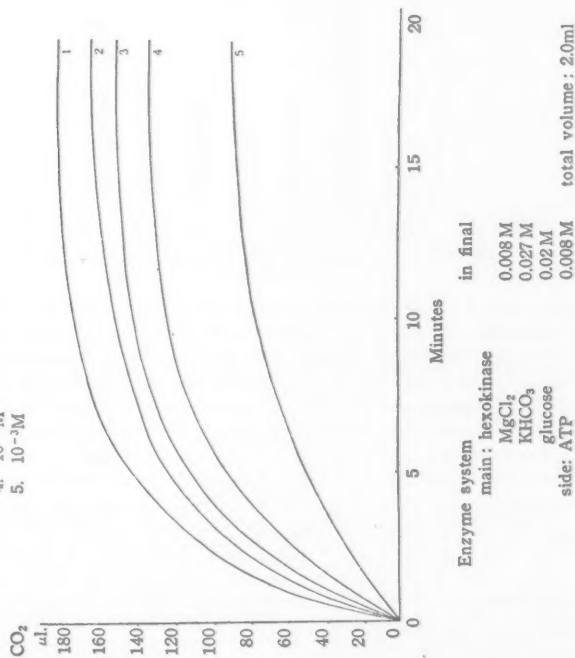


Fig. 11.

Animal hexokinase (prepared by Stein-Cori's method)

1. control
2. in final Xanthurenic acid  $10^{-3}$ M  
ATP in final  $4 \times 10^{-3}$ M

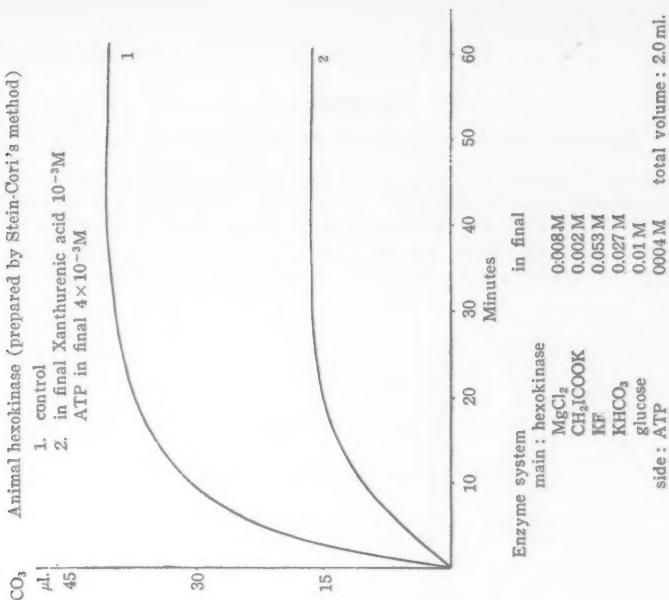


Fig. 12.

## APPENDIX I

## INHIBITORY EFFECT OF INSULIN ON XA PRODUCTION IN THE BODY

To a group of albino rats, 0.1 Gm. of tryptophan and 0.4 Gm. of sodium butyrate were administered, so as to excrete XA in the urine, several animals being used as a control. In parallel with this, we injected various quantities of insulin to the rest of them. The 24-hour urine from each rat of the two sub-groups was collected and the amount of XA was estimated. The results shown in Fig. 13 indicate that only an

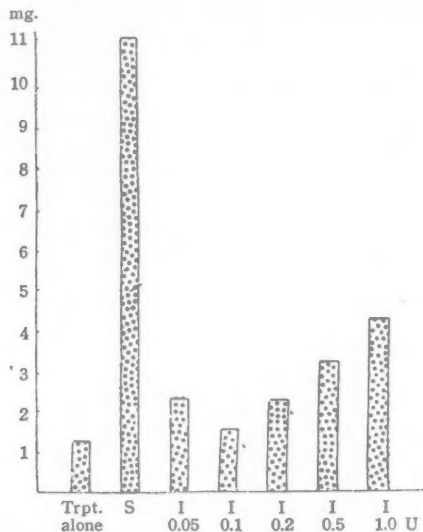


Fig. 13. The amount of XA excreted in 24-hour urine.

appropriate amount of insulin could remarkably depress the excretion of XA and inhibit its production in the body. Furthermore, we succeeded in separating and crystallizing a substance produced presumably in place of XA after administering 0.1 unit of insulin together with tryptophan and butyrate. The amount obtained from the urine of rat was 10 mg., having mp. 258-269°. Since both tests of Kretchey and Jaffé proved to be positive, it is considered to be kynurenic acid. For its identification an absorption spectrum was estimated, comparing with the synthetic sample of kynurenic acid of Roche. As shown in Fig. 14, the curve was proved to be practically identical with the synthesized preparation. This experiment is significant, considering the experiments by Yashiro Kotake and Ichihara showing that kynurenic acid is nothing but a non-toxic, non-effective final product of tryptophan. Presumably kynurenic acid is the only non-toxic metabolite possessing a quinoline ring, and less XA is produced after insulin administration, while kynurenic acid is produced in its place, probably as a result of a sort of anti-toxic action.

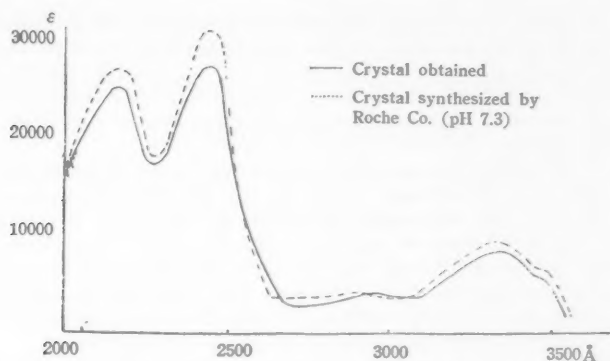


Fig. 14. Absorption spectrum of kynurenic acid.

Tested animals: XA 200 mg./kg. and 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid ethylester 20 mg./kg. injection.

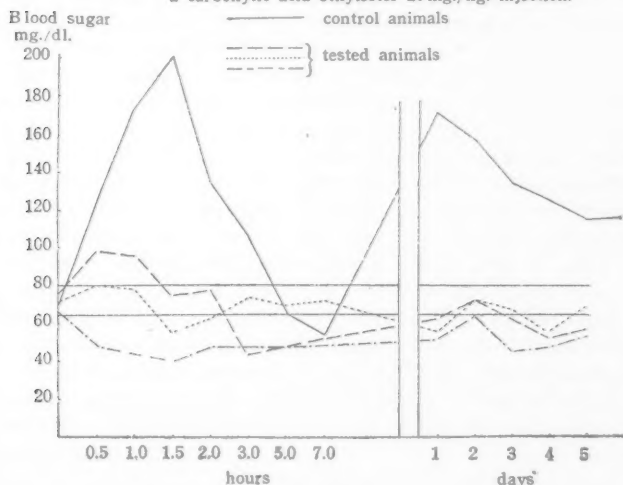


Fig. 15.

## APPENDIX II

ACTION OF 4-HYDROXY-8-METHOXY-QUINOLINE-2-CARBOXYLIC ACID ETHYLESTER AND ITS FREE ACID WHICH ARE INTERMEDIARY PRODUCTS OF SYNTHETIC XA AS THE INHIBITING AGENT AGAINST THE DIABETOGENIC PROPERTY OF XA

A group of albino rats were intraperitoneally injected 200 mg. per kilo of XA as controls. The other group was injected 100 mg. or 20 mg. per kilo of 4-hydroxy-8-

methoxy-quinoline-2-carboxylic acid ethylester and 10 minutes later re-injected intraperitoneally 200 mg. per kilo of XA. Figure 15 shows the variations in the blood sugar level in terms of time.

In each of these test animals, 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid was found to be capable of inhibiting the diabetogenic property of xanthurenic acid in a remarkable degree.

In view of this finding, it is now safe to conclude that for any of the substances with quinoline ring to have its diabetogenic property, free OH radical in the 8 position of quinoline ring is utterly indispensable and that if this OH radical is substituted by methoxy radical, the substance not only loses its diabetogenic property, but also inhibits the diabetogenic action of XA itself. This fact further reveals it to be an indispensable condition that in an experiment for diabetogenic properties using synthetic XA care should be taken that 4-hydroxy-8-methoxy-quinoline-2-carboxylic acid which can so easily be contained is completely eliminated before its actual employment.



# The Significance of Some B-Complex Vitamins in Clinical Chemistry

Noris Siliprandi

THE SIGNIFICANCE of the B vitamins became evident when they were recognized to be fundamental constituents of the coenzymes. The "catalytic activity" of the vitamins could then be explained on the basis of their indispensability for several enzymatic activities. Previously the vitamins were considered to be "catalysts," as very small amounts were required to produce profound changes in biologic systems.

The close relationship existing between vitamins and coenzymes was clearly demonstrated in the 1930s when riboflavin was shown to be a component of the "yellow enzyme" (1). Two years later an essential part of the enzyme system required for the biologic decarboxylation of pyruvic acid was identified as thiamine pyrophosphate (2). By 1935, nicotinamide was demonstrated as being a part of TPN, the coenzyme essential for reactions taking place when glucose is utilized by erythrocytes and yeast (3). In the 1940s vitamin B<sub>6</sub> (4), pantothenic acid (5), and quite recently thioctic acid (6) were added to the list of the B vitamins which possessed coenzyme functions.

All the presently known coenzymes derive from B vitamins (Table 1) and it is now possible to explain the metabolic role of the above-mentioned B vitamins on the basis of their coenzyme function. On the other hand, in all cases the coenzyme appears to be the only metabolically active form for those particular vitamins. The coenzyme function of biotin, folic acid, B<sub>12</sub>, etc., was suggested by many authors (7).

In their excellent book *The Biochemistry of B Vitamins*, Williams *et al.* (8) have developed the modern concepts regarding the catalytic function of B vitamins and their fundamental biochemical significance. In this connection the same authors emphasized the universal occurrence of the B vitamins and their necessity for the life of all organisms as a consequence of the absolute requirement of these factors for all essential "processes which form the foundation upon which life is built."

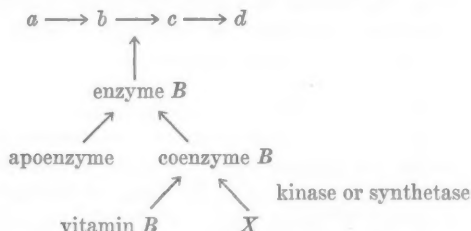
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Adequate discussion of the chemistry and function of the classic coenzymes may be found in the monographs of Novelli and Soodak (7), Neilands and Stumpf (9), and in the symposium which appeared in *Physiological Reviews*, October, 1953.

These views on the significance of B vitamins find their counterpart in human pathology and clinical practice. Since these vitamins accomplish their metabolic role only if previously transformed in the organism into the corresponding coenzymes, metabolic disorders may develop from a deficient intake due to a faulty diet, or it may result from an inadequate capacity of the organism in converting free vitamins into coenzymes.

If in a metabolic sequence the reaction  $b \longrightarrow c$  is dependent on the enzyme  $B$ , the several causes which may affect the efficiency of this enzyme result in an impairment of the metabolic pathway and in an accumulation of the intermediate  $b$  and its precursors. The following diagram illustrates, in the light of our present knowledge, how a typical enzyme  $B$  is built up in the organism and summarizes the conditions required for its formation and efficacy:



( $X$  = orthophosphate or pyrophosphate radical, or adenylic acid derivatives)

The first condition is that apoenzyme and coenzyme, both available in the organism in sufficient amounts, can combine to give rise to the active holoenzyme. No approach to the problem of the availability of the apoenzyme and of its binding ability with coenzyme has so far been made in clinical chemistry.

Availability of the organism for coenzymes depends upon:

1. A sufficient uptake of the corresponding vitamin.
2. The presence in sufficient amounts of nonvitamin moieties ( $X$ ), with which vitamins have to react to form coenzymes. This moiety is represented by an orthophosphoric radical in the case of conversion of riboflavin into FMN and of pyridoxal into pyridoxal-5-phosphate, and by the pyrophosphoric radical for the conversion of thiamine to cocarboxylase. ATP is in all instances the donor of both orthophosphoric and pyrophosphoric radicals. In the case of the synthesis of FAD, DPN, TPN, and CoA,  $X$  is represented by much more complicated compounds, consisting of or including in all cases ATP (see Table 1).

Table 1. BIOSYNTHESIS OF THE COENZYMES DERIVING FROM B VITAMINS

	Reference
1). a). Thiamine + ATP $\xrightleftharpoons{\text{thiaminokinase}}$ AMP + [DPT]	(11, 12)
b). Thiamine + 2 ATP $\xrightleftharpoons{\text{thiaminokinase}}$ 2 ADP + [DPT]	(13)
2). a). Riboflavin + ATP $\xrightleftharpoons{\text{flavokinase}}$ ADP + [FMN]	(14)
b). FMN + ATP $\xrightleftharpoons{\text{FAD synthetase}}$ pyrophosphate + [FAD]	(15)
3). a). NMN + ATP $\xrightleftharpoons{\text{DPN pyrophosphorylase}}$ pyrophosphate + [DPN]	(16)
Mg++	
b). DPN + ATP $\xrightleftharpoons{\text{DPN kinase}}$ ADP + TPN	(17, 18)
Mg++	
4). Pyridoxal + ATP $\xrightleftharpoons{\text{pyridoxal kinase}}$ ADP + [pyridoxal-5-phosphate]	(19)
5). a). Pantoate + alanine + ATP $\xrightleftharpoons{\text{pantothenate synthesizing enzyme}}$ pantothenate + AMP + + pyrophosphate	(19)
b). Pantothenine + ATP $\xrightleftharpoons{\text{pantothenine kinase}}$ phosphopantothenine + ADP	(20)
c). Phosphopantothenine + ATP $\xrightleftharpoons{\text{dephospho-CoA pyrophosphorylase}}$ dephospho-CoA + pyrophosphate	(21)
d). Dephospho-CoA + ATP $\xrightleftharpoons{\text{dephospho-CoA kinase}}$ ADP + [CoA]	(22)
Mg++	

DPT = Diphosphothiamine (cocarboxylase)  
 FMN = Flavinmononucleotide (riboflavinphosphate)  
 FAD = Flavinadenindinucleotide

DPN = Diphosphopyridinnucleotide

TPN = Triphosphopyridinnucleotide

CoA = Coenzyme A

ATP = Adenosintriphosphate

ADP = Adenosindiphosphate

AMP = Adenosinmonophosphate (adenylic acid)

NMN = Nicotinamide mononucleotide

3. Efficiency of the enzymes (kinases or synthetases) which catalyze the transformations of vitamins to coenzymes.

The first of these conditions, i.e., deficiency states of the B vitamins, has been extensively investigated and is well known. B vitamin deficiencies generally occur as a result of an unbalanced diet with submarginal intake of the B vitamins: "primary deficiency" (10). It very frequently happens that more than one vitamin is lacking in the diet. Consequently many of the classic pictures of deficiency are the result of the lack of more than one factor, and

clear-cut clinical cases involving a deficiency of only one nutritional factor are seldom encountered. Moreover, vitamin B deficiency may result from factors other than an inadequate diet, that is, from a "conditioned deficiency" (10). This so-called "conditioned deficiency" may be caused by factors interfering with gastrointestinal absorption of essential factors, or by factors which increase the requirements (23); or it may be due to excessive destruction or excretion.

This topic will not be developed here as a considerable number of excellent biochemical and clinical publications may be consulted by those who are interested in this aspect of the B complex. It is, however, pertinent to remark that all known biochemical features involved in vitamin B deficiency may be explained on the basis of metabolic disorders following the impaired function of the enzyme in which vitamin B is known to participate. Most of the clinical symptoms of deficiency, on the contrary, have not yet been correlated to the enzymatic reactions in which vitamins take place. As Williams *et al.* (24) affirm: "there is no clearly defined positive evidence that any of the typical B vitamins has any indispensable role other than those associated with the specific type of reactions for which it is required."

On the other hand, pathologic conditions are known which, though different in their clinical symptoms, are substantially similar to the classic avitaminoses for their biochemical features. As no vitamin uptake inadequacy could account for the enzymatic deficiency encountered in these diseases, attention has been focused to the above-mentioned processes by which the inactive vitamin is transformed into the active coenzyme.

### THIAMINE

Recent studies on the thiamine metabolism in pancreatic diabetes succeeded in explaining some of the metabolic disorders of this disease as a result of an impaired transformation of thiamine into cocarboxylase. These studies may be quoted here as an example of an approach to the problem of the significance and the role of the B vitamins in a typical metabolic disease.

In 1939, Goodhart and Sinclair (25) conducted a systematic survey of blood cocarboxylase levels in a number of pathologic conditions. They found that, generally speaking, the amount of cocarboxylase in the blood varied directly with the amount of total thiamine and also with the degree of saturation of the tissues with the vitamin. However, a number of exceptions were found to this general rule. Some were related to cases where the blood cell count was greatly increased, for instance, in cases of polycythemia vera and myeloid leukemia. Patients who had recently received intensive therapy with vitamin B<sub>1</sub> over a very short period of time also exhibited high blood thiamine levels, associated with a relatively low blood cocarboxylase content. A group of individuals showing such discrepancy were 3 patients with diabetes, 1 of whom exhibited cardiac failure and 2 diabetic neuritis.

More recently, Foa *et al.* (26) observed that the administration of thiamine to pancreatectomized dogs was followed by an increase of blood cocarboxylase only when insulin was injected at the same time. On the other hand, Markees (27), in a systematic study of blood pyruvate levels in physiologic and pathologic conditions, found that in experimental alloxan diabetes, pyruvate is at normal level; the injected pyruvate, however, disappears from the blood of these animals more slowly than in the normal ones. In diabetic coma the pyruvate level of the blood was found to be considerably increased. These metabolic conditions resemble the principal biochemical features which occur in thiamine deficiency in which blood and urinary pyruvate and lactate are increased, while thiamine and cocarboxylase are decreased.

These findings appeared very interesting in elucidating both the mechanism of the conversion of thiamine into cocarboxylase and some aspects of the pathogenesis of pancreatic diabetes and stimulated us to undertake a number of experimental studies on these problems and on the possible role of insulin in the synthesis of cocarboxylase. In a first series of experiments we found that when alloxan diabetic rats were injected with thiamine pyrophosphate, an immediate fall in the level of blood sugar and a rise in the respiratory quotient and tissue glycogen occurred (28). Injections of large doses of thiamine led to similar effects, except in animals which were severely diabetic (29). Injecting thiamine pyrophosphate in diabetic patients also led to a temporary fall in blood sugar and a rise in the respiratory quotient (30). These preliminary results led us to the hypothesis that, in diabetes, thiamine could not be converted to cocarboxylase. This hypothesis was tested experimentally (31, 32). The cocarboxylase content of the liver of alloxan diabetic animals was first determined and found to be significantly lower than that of nondiabetic animals; the decrease appeared to be proportional to the severity of the diabetes. The effect of thiamine administration was next studied. In normal animals the injection of thiamine was followed by an increase of the liver cocarboxylase; this increase occurred in all the animals tested and exceeded 110 per cent. Diabetic animals, however, treated in the same manner showed a much smaller rise in liver cocarboxylase.

The respiratory quotient of moderately diabetic animals and of severely diabetic animals was determined, and the animals were then injected with thiamine and glucose. Sixty minutes later the respiratory quotient was again determined. A few hours later a further dose of thiamine was administered and the liver cocarboxylase was determined. The animals more severely affected by diabetes failed to show an appreciable increase of the respiratory quotient; in such animals the cocarboxylase content of the liver remained very low, of the same order as that found in the untreated diabetic rats. On the other hand, in animals which showed an increase in the respiratory quotient after the injection of thiamine, the liver cocarboxylase was considerably increased. These results are summarized in Table 2. The findings indicate that

Table 2.

A. Stock rats of both sexes of 200 Gm. approximately were used. Thiamine hydrochloride 20 mg./kg. body weight by intramuscular injection; animals killed by decapitation 30 min. after the injection. Cocarboxylase was determined by the method of Ochoa and Peters (4) with minor adaptations (5)

Rats	No. of animals tested	Liver cocarboxylase ( $\mu\text{g./Gm. wet tissue}$ )	
		Range	Average
Normal	22	7.70-12.91	9.62 $\pm$ 0.23
Diabetic	25	3.24-6.39	4.57 $\pm$ 0.16
Normal + thiamine	15	16.40-24.45	20.49 $\pm$ 0.67
Diabetic + thiamine	15	3.80-11.10	6.69 $\pm$ 0.61

B. Respiratory quotient (R. Q.) measured by the method of Haldane-Margaria (6). Cocarboxylase determined a few hours after the R. Q. and 30 min. after an additional dose (20 mg./kg.) of thiamine

R. Q. of diabetic rats		Liver cocarboxylase ( $\mu\text{g./Gm. wet tissue}$ )
Before	60 min. after thiamine + glucose	
0.65	0.67	3.80
0.74	0.95	13.74

Reproduced from *Nature* 168, 422, 1951.

in alloxan diabetes the phosphorylation of thiamine is partially or completely inhibited and, consequently, it offers an explanation for the high levels of blood  $\alpha$ -ketoacids found in severe pancreatic diabetes.

In order to investigate whether insulin acts on the synthesis of cocarboxylase we have studied the action of this hormone on those forms of alloxan diabetes in which the coenzyme was almost completely inhibited. It was shown that the liver cocarboxylase was increased in diabetic animals treated with thiamine plus insulin. Administration of insulin to diabetic rats not treated with thiamine does not give rise to a significant increase in the cocarboxylase content (33).

Similar results were obtained in human diabetics. Blood cocarboxylase of these patients has been found decreased and such a decrease was in general related to the severity of the diabetes. Except in the most severe cases, administration of insulin restored the blood cocarboxylase to near normal, i.e., to the level of normal subjects (34). These results strongly indicate that in diabetes mellitus insulin acts in the biosynthesis of cocarboxylase and, generally speaking, is necessary for the normal processes of thiamine phosphorylation.

In the light of the above-mentioned observations, it was thought of interest to investigate the possible causes to which the impaired phosphorylation of thiamine in severe pancreatic diabetes might be attributed. According to

the above diagram, these may consist in a phosphate donor deficiency, in a decreased thiaminokinase activity, or in a lack of both factors.

Lipton and Elvehjem (35) and Weil-Malherbe (36) in yeast, and Ochoa (37), Lipschitz *et al.* (38), and Leuthard and Nielsen (39) in animal tissues, demonstrated that ATP is the specific phosphoric donor for thiamine phosphorylation. This has recently been confirmed by Rossi Fanelli and his coworkers (40), who, studying thiamine phosphorylation in the intact rat found that ATP labeled with  $P^{32}$  was a precursor of cocarboxylase.

A decreased level of ATP in the tissues of diabetic animals has actually been demonstrated by Cahn and Houget (41), Baccari (42), and by Cutolo and Siliprandi (43). On the other hand, the administration of  $ATP^{32}$  plus thiamine to diabetic animals did not result in a formation of  $DPT^{32}$ , as was the case in normal animals (44). Consequently it seemed improbable that the decreased content of ATP in diabetic tissues might, alone, be responsible for the thiamine phosphorylation deficiency.

We then turned our attention to the study of the enzyme catalyzing thiamine phosphorylation. A liver fraction capable of catalyzing the transfer of a pyrophosphoric radical from ATP to thiamine has been isolated by Leuthard and Nielsen (39), who called it thiaminokinase. More recently Cerecedo *et al.* (45) obtained this enzyme in a purified form from the intestinal mucosa and from the liver of the rat, and found that both ATP and  $Mg^{++}$  were required for its activity. Using purified thiaminokinase preparations from the liver of alloxan diabetic rats we demonstrated that insulin, previously incubated in rat serum, remarkably enhanced the activity of this enzyme when it was prepared from diabetic animals, but not when obtained from control animals (46). Insulin thus appears to exert a direct action on thiamine phosphorylation through an activation of thiaminokinase. A partial inactivation of this enzyme is, most probably, the cause of the impaired utilization of thiamine as cocarboxylase in pancreatic diabetes.

These investigations resulted in the introduction of cocarboxylase in the therapy of human diabetes. A favorable effect of cocarboxylase administration was observed by Markees (27) in diabetic coma and by ourselves in severe diabetes mellitus (30). Intravenous injections of cocarboxylase (1-1.5 mg. kg. body weight) are now widely used in continental Europe as an efficient coadjuvant of the classic insulin therapy. Cocarboxylase alone is an insufficient therapeutic agent, as the impaired phosphorylation of thiamine is most probably only one of the numerous consequences of reduced insulin efficiency. On the other hand, it is important to observe that, while insulin alone is generally unable to reduce the increased blood cholesterol level, such as is found in diabetes, an associated therapy with insulin and B-complex coenzymes (DPT, FMN, DPN, and pyridoxal-5-phosphate) resulted in an appreciable fall of blood cholesterol level (47). Furthermore, Markees (27) demonstrated that insulin administration did not effect the increased pyruvic acid level in



diabetic acidosis, whereas cocarboxylase administration had a definite effect.

The various gaps still existing in our knowledge of the significance of thiamine and cocarboxylase in pancreatic diabetes will probably be filled when the action of cocarboxylase on oxidative decarboxylation of  $\alpha$ -ketoacids is elucidated. At the moment it is only known that cocarboxylase, together with lipoic acid, DPN, and CoA is an indispensable factor for this fundamental metabolic function, and consequently the therapeutic use of cocarboxylase seems reasonable in other pathologic conditions in which an increased level of  $\alpha$ -ketoacids or their decreased utilization after injection is found.

In a systematic study on several human diseases Markees (27) and Lasch (48) observed a frequent increase of the blood  $\alpha$ -ketoacid level in the following conditions: kidney disease, pregnancy, eclampsia, toxicosis of sucklings, celiac disease, acetone vomiting of the child, hepatitis gravis, cobalt and barbiturate poisoning, X-ray sickness, extreme fatigue, burns, and hypoxemia.

In several of these diseases cocarboxylase therapy has been proved to be useful by different authors: toxicosis of sucklings (49, 50), thyrotoxicosis (51), toxic diphtheria (52), delirium tremens (53), paresthesia and muscle cramps in pregnancy (54), vomiting of pregnancy (55), eclampsia (56) [Feola (57), however, did not find any appreciable action in eclampsia], narcotic and postnarcotic states (58), postoperative states (59), heart failure (60), multiple sclerosis (61), myasthenia gravis (62), progressive muscular dystrophy (63), bronchial asthma (64), and herpes zoster (65).

Very interesting results have recently been obtained by Bracco *et al.* (66) in experimental tuberculosis in guinea pigs, and by Petruccioli and Bossa (67) in human tuberculosis. These authors have found that subjects affected by severe forms of pulmonary tuberculosis resistant to streptomycin treatment, when subjected to combined treatment with streptomycin and cocarboxylase, showed an immediate amelioration in clinical symptoms and in the radiologic picture. This striking action of cocarboxylase in restoring the efficiency of streptomycin tubercular infection is obscure. Three factors, however, have some significance in this connection: (1) cocarboxylase exerts a favorable action on the growth of Koch bacillus in vitro (66), a direct bacteriostatic action of this coenzyme in vivo appearing hardly probable; (2) it is known that accumulation of  $\alpha$ -ketoacids and polycarboxylic acids in pulmonary foci of inflammation favors the growth of Koch bacillus (68), the action of cocarboxylase on pulmonary tuberculosis being, therefore, interpreted as a consequence of a decreased  $\alpha$ -ketoacid accumulation at the side of inflammation; (3) both in guinea pigs experimentally infected with Koch bacillus and in humans with tuberculosis, cocarboxylase administration protects the myocardium from toxic damage due to the infection (69), and a favorable action of cocarboxylase itself on general toxicosis may therefore be considered to take place.

Although some of these therapeutic results cannot be considered as final,



the introduction of cocarboxylase in therapy proved a useful tool for the clinician. It should be remembered that although a condition of shock following large intravenous doses of thiamine has often been observed, no toxic effect has ever been encountered with cocarboxylase. This constitutes a further therapeutic advantage of cocarboxylase over thiamine when parenteral administration of vitamin B<sub>1</sub> is required.

An important aspect of the problem of therapeutic cocarboxylase adequacy is concerned with the capacity of this coenzyme to pass as such through the cell membrane. In a comparative study of the catatorulin effect exerted by thiamine and cocarboxylase on intact cells of thiamine-deficient animals, Banga *et al.* (70) concluded that the free vitamin passes into the cells much more rapidly than its phosphorylated derivative. The notion that cocarboxylase is unable to enter the cells as such, has since been widely accepted. Banga *et al.* (70), and Lohmann and Schuster (71), however, have observed that at higher concentrations, thiamine and cocarboxylase are equally active in the catatorulin test. In a still unpublished paper we have studied the permeability of normal muscular cells to thiamine and cocarboxylase, and have found that both compounds pass through the cell membrane, though a higher concentration and longer equilibration time is required for the latter compound.

It should be pointed out that these results were obtained in *in vitro* studies and therefore cannot be taken as direct evidence of what takes place in the whole animal.

In experiments on living dogs Siliprandi and Laviano (72) demonstrated that the administration of diphosphothiamine is more effective than that of thiamine itself in maintaining the level of blood cocarboxylase higher than in normal conditions for at least 24 hours.

Experiments on the capacity of thiamine P<sup>32</sup>-labeled esters to enter the cells *in vivo* are being carried out in our laboratory at the moment.

### RIBOFLAVIN

As we have noted a good understanding of thiamine function in clinical chemistry has been made possible by the following favorable circumstances: (1) accumulation of a well-defined and easily determinable metabolite, as a consequence of coenzyme inefficiency; (2) possibility of a routine determination of the coenzyme in tissues or in body fluids; and (3) availability of large amounts of the pure coenzyme for clinical trials.

Unfortunately, up to the present time, such has not been the case with the other vitamins of the B complex. Their enzymatic significance in clinical chemistry, therefore, though clearly foreseen, does not rest on substantial experimental and clinical evidence.

Our discussion will, therefore, have to be limited to a few significant data concerning vitamins B<sub>2</sub> and B<sub>6</sub>.

The two coenzymes, FAD and FMN, deriving from riboflavin are well

known both in their biochemical significance and in the mechanism by which they originate from riboflavin (see Table 1). The findings of Bessey *et al.* (73) indicate that riboflavin, like other B vitamins, occurs in animal tissues in a bound form, FAD being the major constituent. The FAD content varies from 90 per cent of the total riboflavin content of the muscle to 70 per cent in the kidney. The remainder occurs mainly as FMN with free riboflavin being present in traces only. The same authors noted that the various forms of riboflavin in tissues is to a great extent independent of the riboflavin intake. In riboflavin deficiency, however, its esterified forms are clearly diminished in the liver and kidney, but not in heart muscle.

These facts indicate that, most probably, riboflavin has, per se, no physiologic function and its significance consists in being the precursor of FAD and FMN.

FAD and FMN, as prosthetic groups of the enzymes designated as flavoproteins, play, in connection with pyridin nucleotides (DPN and TPN) and the cytochrome system, a central role in hydrogen transfer from substrate to oxygen.

Nevertheless, clinical signs of an impaired function of these coenzymes or of the related enzymatic systems are not known. As no particular metabolite is known to accumulate in riboflavin deficiency, we do not dispose of any definite criteria to recognize those conditions in which an accurate investigation of the coenzymes derived from riboflavin is desirable. The clinical literature contains, therefore, only occasional and not coordinated data concerning riboflavin coenzymes.

Thus Suvarnakich *et al.* (74) found increased values of FAD in arteriosclerotic heart disease and hypertension. In exophthalmic goiter there is very little riboflavin excreted in the urine. When riboflavin is given (orally or by injection) it is very rapidly excreted, and after 8 hours the riboflavin content of the urine falls to its previous low level (75). The high excretion, in this case, apparently does not indicate saturated body stores but metabolic failure in riboflavin utilization.

A very interesting systematic study has been made by Kerppola (76) on the blood level of riboflavin and its esterified forms in 505 cases, including 60 normal and 445 patients suffering from various diseases. Determination of free and esterified riboflavin, including FAD and FMN, was made by the microlumiflavin method of Fujita and Matsuura (77). Extremely low values for esterified forms were found by Kerppola in three groups of neurologic diseases: schizophrenia, disseminated sclerosis, and neurocirculatory asthenia. These low values cannot be due to a lack of riboflavin in the diet or to an impaired resorption of vitamin, for the patients showed a normal total riboflavin content. In the opinion of the author the causes may be sought in at least three directions: (1) the conversion of riboflavin into coenzymes may

be deficient, (2) the consumption of riboflavin containing coenzymes may be abnormal, and (3) the splitting of coenzymes could be unusually great.

The investigation of the behavior of the riboflavin coenzymes after administration of free riboflavin and coenzymes themselves could, most probably, give rise to useful information.

Urinary output of free and esterified riboflavin has been extensively studied by Travia *et al.* (78) in several physiologic and pathologic conditions, using the method of Emmerie (79). In chronic liver diseases, in diabetes mellitus, and in Basedow's disease, the ratio of free riboflavin: esterified riboflavin in the urine was found to be increased and the administered riboflavin was found, within a few hours, nearly quantitatively in the urine. It seems, therefore, that in these diseases a disturbance of riboflavin utilization exists.

In alloxan diabetes FAD has been found significantly decreased by Navazio and Siliprandi (80). Riboflavin administration is ineffective in restoring this coenzyme to normal values, while the administration of riboflavin plus ATP or of FMN is, on the other hand, effective. These findings, successively confirmed in *in vitro* experiments with liver cell preparations, give further evidence that ATP is necessary for the biosynthesis of both FMN and FAD (81). The decreased ATP level in diabetic tissues (43) can therefore be held responsible for the reduced FAD content.

The above-mentioned results show that a deficiency of flavin coenzymes may be independent of an inadequate intake of riboflavin. An impairment of their synthesis from riboflavin, therefore, has to be considered as a possible condition which may occur in several metabolic disorders.

### VITAMIN B-6

In the case of thiamine and riboflavin, it has been possible to give significant examples of an impaired formation of coenzymes from free vitamins; vitamin B<sub>6</sub>, on the other hand, offers a very interesting example of another mechanism by which coenzymes, although formed in the normal way, may be inhibited in their specific functions.

It is known that pyridoxal-5-phosphate is the coenzyme of vitamin B<sub>6</sub>. The other members of this group —pyridoxine, pyridoxal, pyridoxamine, and pyridoxamine phosphate— owe their vitamin activity to the ability of the organism to convert them into the enzymatically active form: pyridoxal-5-phosphate. This coenzyme is concerned with the activity of a wide variety of enzyme systems catalyzing the following reactions: amino acid decarboxylation, transamination, racemization, synthesis of tryptophan from indole and serine, and degradation of tryptophan.

The degradation of tryptophan to nicotinic acid, as Dalgliesh has recently pointed out (82), is not only dependent upon vitamin B<sub>6</sub> factors, but it is markedly affected by vitamin B<sub>1</sub> and B<sub>2</sub>. Vitamin B<sub>6</sub> deficiency, however, determines, as one of the earliest symptoms, a derangement in tryptophan

metabolism leading to an increased excretion of urinary xanthurenic acid. As xanthurenic acid is a very readily determinable metabolite, an impaired function of vitamin B<sub>6</sub> coenzyme becomes easily detectable.

It has recently been shown that patients given isonicotinic acid hydrazide (INAH) for the treatment of tuberculosis, developed a peripheral neuritis and other symptoms which closely resemble those of typical vitamin B<sub>6</sub> deficiency. Such patients were also found to excrete greater amounts of xanthurenic acid and of vitamin B<sub>6</sub> apparently in a conjugate form including INAH (83) in their urine.

These observations have been confirmed by in vitro experiments on pyridoxal-5-phosphate dependent enzymes. It has been reported that INAH inhibits *E. coli* arginine decarboxylase, tryptophanase, glutamate-oxaloacetate transaminase (84), and quite recently it was shown that glutamate-pyruvate transaminase (85), prepared from animal liver, was inhibited. Inhibition is reversed by pyridoxal-5-phosphate, pyridoxamine-5-phosphate, and also by pyridoxine, which is presumably converted to pyridoxal-5-phosphate in the cells.

To explain these findings it has been suggested (86) that INAH exerts its primary competitive effect on the vitamin B<sub>6</sub> group by competing with pyridoxal-5-phosphate for the apoenzyme. No experimental evidence for this interpretation has, however, so far been provided.

Recent experiments by Mauron and Bujard (87) on the antagonism existing between INAH, Cu<sup>++</sup>, and pyridoxal in transamination processes supported the hypothesis that INAH forms complexes with pyridoxal-5-phosphate; hence pyridoxal-5-phosphate is unable to exert its normal physiologic function. The findings of Mauron and Bujard agree, on the other hand, with the recent demonstration of Fasella *et al.* (88) that pyridoxal-5-phosphate in chemical transamination couples with amino acids to form intermediate compounds, which have been isolated and identified as Schiff-base chelates between pyridoxal-5-phosphate, aminoacids, and a bivalent or a trivalent ion. A blocking effect of INAH on pyridoxal-5-phosphate seems, therefore, to be the most probable mechanism.

## CONCLUSIONS

The data we have considered, although obtained from three vitamins only, are sufficient to emphasize that the enzymatic function of the vitamins can be impaired by one of the following conditions:

- 1). Deficient intake of vitamins with the diet.
- 2). Interference with gastric or intestinal absorption.
- 3). Impaired conversion of free vitamins into the corresponding coenzymes.
- 4). Coenzyme blockage.

In all these instances the study of coenzymes in the organism has a definite practical significance. The determination of coenzyme activity in the tissues

or in red blood cells constitutes, according to Westenbrink (89), the more convenient way of revealing not only a deficiency of coenzymes themselves, but also of the corresponding vitamins. According to the same author, a decreased level is in fact the first sign of a deficiency of the corresponding vitamin.

A further condition which should be taken into consideration is represented by disturbances of the apoenzyme synthesis, such as is postulated by Esser and Schmengler (90) for Sjögren's syndrome.

According to Vannotti (91), the clinical features which follow a vitamin-function impairment are essentially the same whatever their source of origin. This author describes a "syndrome d'insuffisance des catalyseurs biologiques" which, analogously to the hypovitaminosis due to a faulty diet, is characterized by general metabolic disturbances, cutaneous symptoms (hyperkeratosis, pigmentation, eczema), mucosa alterations (atrophy, dryness, glossitis, stomatitis, gastritis, rhagades), neuritis, myelosis, liver steatosis, and anemia. This syndrom is attributed by the author to an inefficiency of the cellular enzymatic systems due to an impaired conversion of free vitamins into coenzymes. Free vitamins, in fact, are in this case an ineffective therapeutic tool, while coenzyme administration results in an evident amelioration.

In our opinion, however, no definite clinical conclusion can as yet be drawn. Moreover it has to be observed that when the organism is incapable of an adequate transformation of the vitamins into their coenzymes, it is very probable that other metabolic processes, as in the case of diabetes mellitus, are contemporaneously impaired.

The story of the coenzymes deriving from the vitamin B complex in normal and pathologic subjects is as yet incomplete, but the results obtained in this field are sufficiently promising to merit further research in this aspect of clinical chemistry.

## REFERENCES

1. Warburg, O., and Christian, W., *Biochem. Z.* **254**, 428 (1932).
2. Lohman, K., and Schuster, P., *Biochem. Z.* **294**, 112 (1934).
3. Warburg, O., and Christian, W., *Biochem. Z.* **275**, 112 (1934).
4. Gunzalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.* **155**, 685 (1944).
5. Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C., and Guirard, B. M., *J. Biol. Chem.* **167**, 869 (1947).
6. Reed, L. J., DeBusk, B. G., Gunzalus, I. C., and Schnakenberg, G. H. F., *J. Am. Chem. Soc.* **73**, 5920 (1951).
7. Novelli, G. D., and Soodak, M., *Biochemistry and Physiology of Nutrition*, New York, Acad. Press, 1953, Vol. II, p. 326.
8. Williams, R. J., Eakin, R. E., Beerstecher, E., Jr., and Shive, W., *The Biochemistry of B Vitamins*, New York, Reinhold, 1950.
9. Neilands, J. B., and Stumpf, P. K., *Outlines of Enzyme Chemistry*, New York, Wiley, 1955.
10. Bicknell, F., and Prescott, F., *The Vitamins in Medicine*, London, Heinemann, 1953, p. 223.

11. Weil-Malherbe, H., *Biochem. J.* **33**, 1997 (1939).
12. Steyn-Parvé, E. P., *Biochem. et Biophys. Acta* **8**, 310 (1952).
13. Siliprandi, N., Boffi, V., and Lucarelli, A., *Il Farmaco* **10**, 179 (1955).
14. Kearney, E. B., and Englard, S., *J. Biol. Chem.* **193**, 821 (1951).
15. Schrecker, A. W., and Kornberg, A., *J. Biol. Chem.* **182**, 795 (1950).
16. Kornberg, A., *J. Biol. Chem.* **182**, 779 (1950).
17. Kornberg, A., *J. Biol. Chem.* **182**, 805 (1950).
18. Wang, T. F., and Kaplan, N. O., *J. Biol. Chem.* **206**, 311 (1954).
19. Maas, W. K., and Novelli, G. D., *Arch. Biochem. and Biophys.* **43**, 236 (1953).
20. Levintow, L., and Novelli, G. D., *J. Biol. Chem.* **207**, 761 (1954).
21. Hoagland, M., and Novelli, G. D., *J. Biol. Chem.* **207**, 767 (1954).
22. Wang, T. P., and Kaplan, N. O., *J. Biol. Chem.* **206**, 311 (1954).
23. Gruber, M., *Bioch. et Biophys. Acta* **10**, 136 (1953).
24. Williams, R. J., Eakin, R. E., Beerstecher, E., Jr., and Shive, W., *The Biochemistry of B Vitamins*, New York, Reinhold, 1950, p. 99.
25. Goodhart, R., and Sinclair, H. M., *J. Biol. Chem.* **132**, 11 (1940).
26. Foà, P. P., Smith, J. A., and Weinstein, H. R., *Arch. of Biochem.* **13**, 449 (1947).
27. Markees, S., *Schweiz. Med. Wochensh.* **81**, 1145 (1951).
28. Siliprandi, D., and Siliprandi, N., *Archivio di Scienze Biologiche* **35**, 453 (1951).
29. Siliprandi, N., *Acta Vitaminologica* **4**, 249 (1950).
30. Siliprandi, N., and Traverso, R., *Il Farmaco* **5**, 655 (1950).
31. Siliprandi, D., and Siliprandi, N., *Acta Vitaminologica* **5**, 3 (1951).
32. Siliprandi, D., and Siliprandi, N., *Nature* **168**, 422 (1951).
33. Siliprandi, D., and Siliprandi, N., *Nature* **169**, 329 (1952).
34. Siliprandi, N., and Navazio, F., *Acta Med. Scand.* **117**, 149 (1952).
35. Lipton, M. A., and Elvehjem, C. A., *Cold Spring Harbor Symp. quant. Biol.*, **7**, 184 (1939).
36. Weil-Malherbe, M., *Biochem. J.* **33**, 1997 (1939).
37. Ochoa, S., *Biochem. J.* **33**, 1262 (1939).
38. Lipschitz, M. A., Potter, V. R., and Elvehjem, C. A., *Biochem. J.* **32**, 474 (1938).
39. Leuthard, F., and Nielsen, H., *Helv. Chim. Acta*, **35**, 1196 (1952).
40. Rossi Fanelli, A., Siliprandi, N., Fasella, P., Siliprandi, D., and Salvetti, M., *Experientia* **10**, 73 (1954).
41. Cahn, H., and Houget, J., *C. r. Acad. Sci. Paris* **203**, 354 (1936).
42. Baccari, V., *Boll. Soc. it. Biol. Sper.* **25**, 356 (1949).
43. Cutolo, E., and Siliprandi, N., *Experientia*, **8**, 24 (1952).
44. Fasella, P., and Siliprandi, N., Unpublished results.
45. Cerecedo, L. R., Eich, S., and Bresnick, E., *Biochem. et Biophys. Acta* **15**, 144 (1954).
46. Siliprandi, N., Boffi, V., and Lucarelli, A., *Nature* **176**, 219 (1955).
47. Lucidi, A., and Giustiniani, A., *Minerva Medica* **47**, 934 (1956).
48. Lasch, F., *Deut. Med. Wochenschr.* **78**, 975 (1953).
49. Duschl, K., *Kinderärztliche Praxis* **21**, 477 (1953).
50. Goffart, P., Marin, A., and Pollard, G., *Bull. d. l'Hôpital Civil de Charleroi* **4**, 197 (1953).
51. Pico, A., and Dogliotti, G., *Minerva Medica* **45**, 30 (1954).
52. Lasch, F., *Ann. Paediat.*, **178**, 333 (1952).
53. Galian, U., and Gamma, G., *Giorn. psichiatria* **82**, 306 (1954).
54. Lacomme, M., Lepage, R., and Gueguen, J., *Bull. Gynécol. et Obstétr.* **4**, 230 (1952).
55. Krafft, H. C., *Gynaecologia* **135**, 117 (1953).
56. Käser, O., *Z. Geburtsh. u. Gynäk.* **139**, 1 (1953).
57. Feola, M., *Arch. Ostetr. Ginecol.* **58**, 264 (1954).

58. Hügin, W., *Der Anaesthesist* **2**, 193 (1953).
59. Albano, V., and Martino, G., *Pathologica* **45**, 419 (1953).
60. Kleeberg, J., and Gitelson, S., *J. Clin. Path.* **7**, 116 (1954).
61. Ervenich, P., and Krueger, R., *Ärtl. Forsch.* **9**, 189 (1955).
62. De Flora, G., *Acad. Med.* **68**, 102 (1952).
63. Luzzato, A., and Lodigiani, E., *Min. Med.* **45**, 1 (1954).
64. Larizza, P., and Bianchi, G., *Min. Med.* **45**, 74 (1954).
65. Tinozzi, C. C., *Dermatologia III*, 451 (1951).
66. Arnold, R., *Angew. Med.* **1**, 168 (1956).
67. Bracco, M., and Ballerini, G., *Annali del Villaggio di Sondalo* **2**, 371 (1954).
68. Dubos, R., *J. exp. Med.* **98**, 145 (1953).
69. Curti, P. C., personal communication.
70. Banga, S., Ochoa, S., and Peters, R. A., *Biochem. J.* **33**, 1109 (1939).
71. Lohmann, K., Schuster, P., *Biochem. Z.* **294**, 188 (1937).
72. Siliprandi, D., Laviano, F., *Experientia* **9**, 420 (1953).
73. Bessey, O. A., Lowry, O. H., and Love, R. H., *J. Biol. Chem.* **180**, 755 (1949).
74. Suvarnakich, K., Mann, G. V., and Stare, F. J., *J. Nutr.* **47**, 105 (1952).
75. Shapiro, Y. E., *Ter. Arkh.* **25**, 48 (1953).
76. Kerppola, W., *Acta Medica Scand.* **153**, 33 (1955).
77. Fujita, A., and Matsuura, K., *J. Biochem. (Tokyo)* **37**, 445 (1950).
78. Travia, L., Pelosio, C., and Topi, G. C., *Acta Vitaminologica* **6**, 66 (1952).
79. Emmerie, A., *Rec. trav. Chim. Pays Bas* **38**, 290 (1939).
80. Navazio, F., and Siliprandi, N., *Experientia* **11**, 280 (1955).
81. Siliprandi, N., Navazio, F., and Fioretti, P., *Experientia* **11**, 497 (1955).
82. Dalglish, C. E., *British Med. Bull.* **12**, 49 (1956).
83. Biehl, J. P., and Vilter, R. W., *Proc. Soc. exptl. Biol. and Med.* **85**, 389 (1954).
84. Yoneda, M., and Asano, N., *Science* **117**, 277 (1953).
85. Meister, A., and Downey, P. F., *Proc. Soc. exptl. Biol. and Med.* **91**, 49 (1956).
86. Lichstein, H. C., *Proc. Soc. exptl. Biol. and Med.* **88**, 519 (1955).
87. Mauron, J., and Bujard, E., *Résumés des Communications 3<sup>e</sup> Congrès Intern. de Biochimie*, pag. 34, 1955.
88. Fasella, P., Lis, H., Siliprandi, N., and Baglioni, C., *Biochim. et Biophys. Acta*, **23**, 417 (1957).
89. Westenbrink, H. G. K., *Exposés Annuels de Biochimie Médical* **17**, 81 (1955).
90. Esser, H., and Schmengler, V. E., *Ärztliche Forschung* **5**, 313 (1951).
91. Vannotti, A., *Vitamines et enzymes (Considerations cliniques)*, in "Convegno sulle Vitamine" p. 492, Milano, 1953.

# Les Acides du Cycle Tricarboxylique en Chimie Clinique

Jo Nordmann et Roger Nordmann

## INTRODUCTION

Le cycle tricarboxylique est l'un des phénomènes métaboliques les plus importants de l'organisme. C'est, en effet, à la fois le fournisseur essentiel de l'énergie des organismes et c'est aussi la plaque tournante principale où s'intègrent les métabolismes des glucides, des lipides et des protides. Krebs (75) a résumé récemment les principales étapes de la découverte de cet ensemble métabolique. Le schéma actuel du cycle tricarboxylique est représenté dans la Fig. 1.

Malgré son rôle de premier plan, il subsiste bien des inconnues concernant le cycle tricarboxylique. La conception même de ce cycle résulte essentiellement d'expériences réalisées *in vitro*. Il semble cependant d'un grand intérêt d'étudier la présence et le rôle des composants du cycle citrique *in vivo*. C'est ce que nous tenterons de faire au cours de cet exposé, en envisageant d'abord la présence des acides de ce cycle dans les liquides biologiques, en discutant ensuite de leur rôle physiologique, puis en étudiant le mode d'action de l'ensemble du cycle tricarboxylique *in vivo*, à l'aide des épreuves de charge,<sup>1</sup> pour terminer enfin sur l'étude de certaines conditions pathologiques et de leur influence sur les constituants du cycle tricarboxylique.

## LES ACIDES DU CYCLE TRICARBOXYLIQUE DANS LES LIQUIDES BIOLOGIQUES: METHODES D'ETUDE ET RESULTATS

### LES ACIDES DU CYCLE TRICARBOXYLIQUE DANS L'URINE

Isolé en 1784 par Scheele à partir du jus de citron, l'acide citrique a été considéré comme un acide typiquement végétal jusqu'à sa mise en évidence

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<sup>1</sup>Nous entendrons par épreuves de charge l'administration d'un métabolite et l'étude des modifications qui en résultent. Au cours de cet exposé, nous emploierons indifféremment le nom de l'acide ou du sel, par exemple citrate ou acide citrique, succinate ou acide succinique, etc.



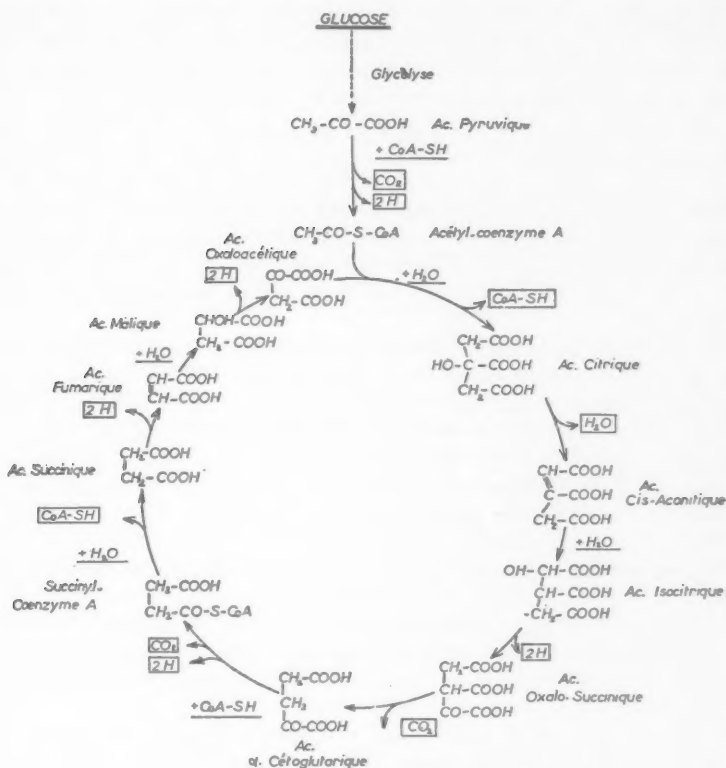


Fig. 1. Schéma du cycle tricarboxylique.

dans le lait en 1888 (59). Considéré par Thünberg comme un métabolite normal depuis 1910, il fut décelé chez l'animal dans l'urine après administration de citrate en 1916 (145) et, peu après, dans l'urine humaine normale par Amberg et McClure (2).

Son excretion a été étudiée ensuite par de nombreux auteurs (cf. en particulier 3, 13, 54, 78, 80, 94, 101, 125, 156, 157, 187). L'élimination quotidienne chez l'homme est, selon les auteurs de 200 à 1000 mg. (125) ou 1000 à 1500 mg. (101), soit 100 à 750  $\mu$ g. par cc. d'urine (187).

L'acide  $\alpha$ -cétoglutarique a été décelé tout d'abord en 1936 (160) dans l'urine de rats atteints d'avitaminose B (principalement B<sub>1</sub>), puis trouvé également chez des rats sains, où sa concentration urinaire est de 2 à 10 mg. pour 100 cc. (78).

La présence de cet acide dans l'urine humaine a été rapportée par Krebs (70), qui trouva chez trois sujets normaux une élimination de 14.5 à 25.7 mg. par jour. Peu après, des chiffres physiologiques plus élevés, soit 20 à 60 mg. par jour, furent rapportés à la suite de dosages effectués dans 10 cas (77).

L'élimination urinaire de l'acide  $\alpha$ -céto-glutarique a été étudiée plus récemment par des techniques chromatographiques (24, 153, 168, 201, 202), les taux normaux étant en moyenne de 20.5 mg. par jour (24) (moyenne de 5 déterminations chez des sujets sains) ou 18.9 mg. par jour (202). Nous-mêmes trouvons en moyenne, par une technique dérivée de celle de El Hawary et Thompson (36), 23 mg. chez l'homme normal et 0.68 mg. chez le rat (rat mâle adulte de souche Sprague-Dawley mis à un régime semi-synthétique décrit ailleurs (108).

L'acide succinique a été décelé tout d'abord dans l'urine du rat et du lapin par Krebs, Salvin et Johnson (71). Dans les quelques cas étudiés par ces auteurs, l'élimination quotidienne chez le rat était de 0.64 à 1.46 mg. Krusius (78, p. 143) a trouvé chez un lapin une élimination de 20.8 mg. par jour.

L'acide succinique a été déterminé également dans une urine humaine normale en 1939, l'élimination trouvée dans ce cas étant de 31 mg. par 24 heures (162). Selon des travaux ultérieurs (180, 181, 193), l'élimination quotidienne normale n'est que de 2 à 12 mg.

*Les travaux que nous venons de citer avaient permis la mise en évidence dans l'urine de trois des acides du cycle tricarboxylique: les acides citrique,  $\alpha$ -céto-glutarique et succinique. Les autres acides de ce cycle n'avaient, par contre, à notre connaissance, jamais été déterminés dans l'urine normale. Pour combler cette lacune, nous avons élaboré une technique permettant la caractérisation de tous les acides stables du cycle tricarboxylique.*

Nous avons fait appel, dans ce but, à la chromatographie sur papier. On connaît la masse de travaux consacrés à la détermination des acides aminés des liquides biologiques par chromatographie sur papier. Cette détermination est rendue relativement aisée par l'existence de méthodes de révélation sélectives (en particulier par la ninhydrine). De plus l'élimination des substances interférentes n'est pas toujours indispensable avant la chromatographie des acides aminés; lorsqu'elle s'avère nécessaire, elle est facilitée par le caractère amphotère de ces acides.

La situation est plus complexe pour les acides du cycle tricarboxylique et, d'une façon générale, pour les acides organiques non aminés, car il n'existe aucune méthode spécifique de révélation de ces acides et l'élimination des substances interférentes préalable à la chromatographie pose des problèmes plus ardu. Malgré ces difficultés, nous avons pensé que seule la chromatographie sur papier permettrait de réaliser une technique de caractérisation suffisamment simple et rapide pour pouvoir être utilisée en chimie clinique. Les séparations chromatographiques des acides du cycle tricarboxylique sur

colonne de résine d'échange anionique (19) ou de silice sont, en effet, des opérations longues, nécessitant une assez grande quantité du mélange à séparer, et sont donc difficilement utilisables en série pour les liquides biologiques, tels que le sang ou le liquide céphalo-rachidien.

La technique que nous avons mise au point s'applique en principe à l'ensemble des liquides biologiques. Nous indiquerons rapidement les points essentiels de son application à l'urine.

Pour minimiser l'influence des variations de dilution de l'urine, nous utilisons toujours le volume d'urine contenant une quantité déterminée de créatinine (soit, dans la plupart des cas, 2 mg.; lorsque la teneur en acides organiques est supposée élevée, par exemple pour les urines recueillies au cours des épreuves de charge, cette quantité est réduite à 1 ou 0.5 mg.).

L'élimination des substances interférentes est réalisée par passage de ce volume d'urine, suivi de 15 cc. d'eau sur une colonne de résine d'échange anionique fortement basique dans le cycle formiate (colonne de 40x4 mm. de Dowex 2, X 10, la taille des particules étant de 150 à 200 mesh, la vitesse de passage de 0.4 cc./minute). Notons que l'on obtient des résultats sensiblement analogues en utilisant une résine faiblement basique, comme la Deacidite (124).

Dans nos conditions, les cations, la plupart des ampholytes et les substances non ionisées ne sont pas retenues sur la colonne (109) et passent dans l'effluent, qui est rejeté. Les anions sont, au contraire, retenus en totalité sur la colonne et en sont élués dans un second temps par l'acide formique. Nous avons étudié de nombreuses modalités d'élution (115), dont aucune ne permet l'élution totale des acides organiques sans élution parallèle d'une partie des anions minéraux retenus également sur la colonne. La technique qui nous a paru la plus satisfaisante est l'élution par 10 cc. d'acide formique 12N, qui libère la totalité des anions organiques, ainsi qu'une partie des phosphates et sulfates et des traces de chlorures. L'intérêt de l'utilisation de l'acide formique pour l'élution réside dans sa volatilité, qui rend aisée son élimination avant la réalisation de la chromatographie sur papier. Cette élimination de l'acide formique est réalisée par concentration de l'éluat dans l'éthuve à 40° en présence d'un mélange de soude et de chlorure de calcium (19).

Le résidu est alors chromatographié en deux dimensions sur Whatman 1. Le solvant alcalin utilisé pour la première dimension est composé de: éthanol absolu (80 volumes), ammoniac 22° Bé soit 21.6% de  $\text{NH}_3$  (5 volumes), eau (15 volumes). Le second solvant, acide, est composé de propanol primaire (50 volumes), eucalyptol (50 volumes), acide formique 98 p. 100 (20 volumes), eau (quantité juste suffisante pour obtenir le premier trouble persistant). L'eucalyptol contenu dans le solvant acide favorise la volatilisation de l'acide du solvant lors du séchage du chromatogramme (26). La révélation est effectuée dans la plupart des cas par un indicateur de pH (habituellement le vert de bromocrésol).

On voit apparaître dans ces conditions une vingtaine de spots sur les chromatogrammes correspondant au volume d'urine contenant 2 mg. de créatinine. Nous avons réalisé l'identification de ces spots à l'aide de techniques, dont le détail a été exposé ailleurs (Nordmann, *et al.* [116]) et qui comprennent en particulier l'étude du comportement chromatographique d'une centaine d'acides témoins et l'utilisation d'une vingtaine de méthodes de révélation différentielle.

Tous les spots, à l'exception d'un seul, ont ainsi été identifiés. Nous en donnons le schéma dans la Figure 2, qui comporte 21 spots, correspondant aux acides suivants :

- Spot 1—Acide fumarique
- Spot 2—Acide m-hydroxybenzoïque
- Spot 3—Acide aconitique
- Spot 4—Acide glutarique
- Spot 5—Acide adipique
- Spot 6—Non identifié
- Spot 7—Acide  $\beta$ -hydroxybutyrique
- Spot 8—Acide hippurique
- Spot 9—Acide succinique
- Spot 10—Acide lactique
- Spot 11—Acide  $\alpha$ -cétoglutarique
- Spot 12—Acide glycolique
- Spot 13—Phénylacétylglutamine
- Spot 14—Acide malique
- Spot 15—Acide pyrrolidonecarboxylique
- Spot 16—Acide citrique
- Spot 17—Acide tartrique
- Spot 18—Acide glucuronique
- Spot 19—Acide chlorhydrique
- Spot 20—Acides oxalique + phosphorique + sulfurique
- Spot 21—Acide urique

*Tous les acides stables du cycle tricarboxylique, à l'exception de l'acide isocitrique, qui est confondu avec l'acide citrique, apparaissent donc sur ces chromatogrammes urinaires, en particulier les acides aconitique, fumarique et malique, dont la présence dans l'urine humaine normale n'avait pas été décrite jusqu'ici. L'absence des acides oxaloacétique et oxalosuccinique n'a rien de surprenant, étant donné la labilité de ces acides, qui se dégradent lors de l'élimination des substances interférentes ou de la chromatographie elle-même.*

Nous signalerons que l'acide aconitique donne une coloration rouge sur les chromatogrammes pulvérisés avec du p-diméthylamino-benzaldéhyde dans l'anhydride acétique (Nordmann, du Ruisseau et Nordmann, [117]). Cette technique de révélation, qui colore en jaune orange le spot correspondant à

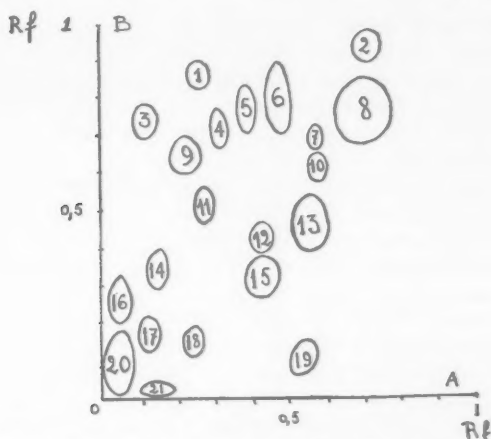


Fig. 2. Schéma des Spots Trouvés dans les Urines Humaines Normales (Volume d'Urine Contenant 2 mg. de Creatinine) (Nordmann *et al.* [113]). *A*, phase solvante alcaline: éthanol, 80 volumes; ammoniac 22° B<sub>é</sub>, 5 volumes; eau, 15 volumes. *B*, phase solvante acide: *n*-propanol, 50 volumes; eucalyptol, 50 volumes; acide formique 98%, 20 volumes; eau, quantité juste suffisante pour obtenir le premier trouble persistant.

Spots: Spot 1, Acide fumarique	Spot 11, Acide $\alpha$ -cétoglutarique
Spot 2, Acide <i>m</i> -hydroxybenzoïque	Spot 12, Acide glycolique
Spot 3, Acide aconitique	Spot 13, Phénylacétylglutamine
Spot 4, Acide glutarique	Spot 14, Acide malique
Spot 5, Acide adipique	Spot 15, Acide pyrrolidonecarboxylique
Spot 6, Non identifié	Spot 16, Acide citrique
Spot 7, Acide $\beta$ -hydroxybutyrique	Spot 17, Acide tartrique
Spot 8, Acide hippurique	Spot 18, Acide glucuronique
Spot 10, Acide lactique	Spot 19, Acide chlorhydrique
Spot 9, Acide succinique	Spot 20, Acides oxalique + phosphorique + sulfurique
	Spot 21, Acide urique

l'acide hippurique (45), est d'une grande utilité pour l'identification des spots, d'autant plus qu'elle peut être appliquée sur des chromatogrammes préalablement révélés par le vert de bromocrésol.

Alors que les spots sont habituellement réguliers et arrondis, celui de l'acide citrique (spot 16) est généralement allongé ou même confondu sous forme d'une traînée avec le spot 20.

Il importe de faire deux remarques à propos du schéma indiqué.

La première est que nous y avons fait figurer les spots que nous avons observés le plus fréquemment sur plus de 2,000 chromatogrammes d'urine humaine. Nous ne saurions cependant, bien entendu, exclure la présence d'autres acides dans l'urine, mais ces derniers n'apparaissent pas en général sur nos chroma-

togrammes, soit qu'ils soient perdus lors de l'élimination des substances interférentes ou lors de la chromatographie elle-même (acides volatils, etc.), soit que leur concentration dans l'urine soit habituellement trop faible pour donner lieu à un spot visible. Cette remarque s'applique en particulier aux acides phénoliques, dont on peut caractériser une grande variété par des techniques spéciales (6).

Le second point est que les spots décrits n'apparaissent pas régulièrement sur tous les chromatogrammes (Fig. 3, p. 469). Parmi les plus constants, nous citerons ceux correspondant aux acides citrique, succinique, malique, hippurique, pyrrolidonecarboxylique (65), ainsi qu'à la phénylacétylglutamine et enfin le spot non encore identifié. La phénylacétylglutamine ne se rencontre pas sur les chromatogrammes d'urine de rat et de lapin que nous avons également effectués. On sait qu'il s'agit du produit de détoxication de l'acide phénylacétique, qui ne se rencontre que chez l'homme (179) et le chimpanzé, (136).

La méthode que nous avons mise au point permet non seulement une identification qualitative, mais également, pour certains acides du moins, une détermination quantitative, que nous envisagerons plus loin.

#### LES ACIDES DU CYCLE TRICARBOXYLIQUE DANS LE SANG

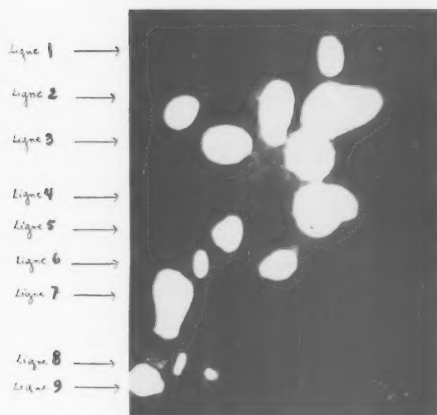
L'*acide citrique* a été identifié tout d'abord dans le sang par Benni, Schersten et Ostberg (10). Sa concentration physiologique dans le sérum, déterminée par la technique enzymatique de Thunberg, est de 1.5 à 3.75 mg. pour 100 cc. (80, 148). La concentration plasmatique est plus élevée que celle du sang total (respectivement 2.5 et 1.5 mg. pour 100 cc.) (183, 187); cette différence est due à la faible quantité d'acide citrique contenue dans les hématies humaines (101, 107). Les hématies du chien seraient, au contraire, riches en acide citrique (137), tandis que celles du lapin et du chat contiendraient 5 à 6 fois moins d'acide citrique que le plasma (94).

De nombreux travaux ont été consacrés au cours des dernières années à la technique de dosage de l'acide citrique sanguin (16, 40, 46, 90, 101, 102, 142, 175, 176, 177, 191).

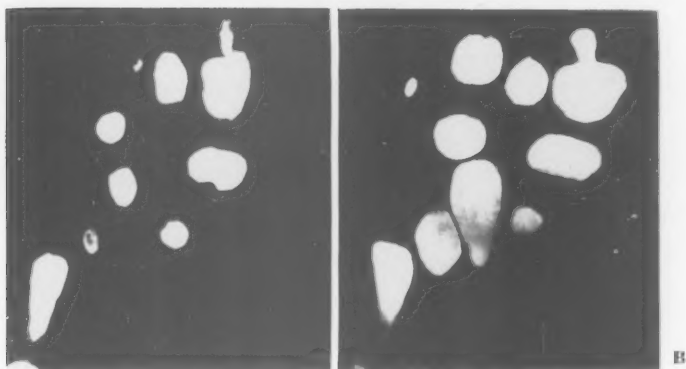
La présence de l'*acide  $\alpha$ -cétoglutarique* a été notée par Krebs (70) dans le sérum de deux malades grâce à une technique enzymatique complexe. Des méthodes colorimétriques plus simples ont été décrites peu après (44); leur spécificité est cependant médiocre, malgré les perfectionnements apportés (50, 68).

Les méthodes chromatographiques actuelles (l'acide  $\alpha$ -cétoglutarique étant dans la plupart des cas chromatographié à l'état de 2,4 dinitrophénylhydrazone) permettent par contre des dosages précis et spécifiques (12, 24, 36, 47, 79, 153, 201).

Les résultats de ces dosages varient quelque peu selon les auteurs, la concentration physiologique de l'acide  $\alpha$ -cétoglutarique dans le sang total humain étant de 0.21 mg. pour 100 cc. (24) (moyenne de 5 déterminations seulement),



**Fig. 3.** Photographie d'un Chromatogramme des Acides Organiques d'une urine Humaine Normale (Nordmann *et al.* [117]). De gauche à droite: *Ligne 1*, acide m-hydroxybenzoïque; *Ligne 2*, acide aconitique; spot non identifié; acide hippurique; *Ligne 3*, acide succinique; acide lactique; *Ligne 4*, phénylacétylglutamine; *Ligne 5*, acide  $\alpha$ -cétoglutarique; *Ligne 6*, acide malique; acide pyrrolidonecarboxylique; *Ligne 7*, acide citrique; *Ligne 8*, acide tartrique; acide glucuronique; *Ligne 9*, spot phosphorique + sulfurique + oxalique.



**Fig. 4.** Chromatogramme des Acides Organiques d'un Sujet Sain Avant ("A") et Aussitôt Apres ("B") Perfusion de Succinate (Nordmann *et al.* [113]). De gauche à droite et de haut en bas: *A*, 1<sup>ère</sup> ligne: Acide fumarique; spot inconnu; acide hippurique (l'appendice situé au haut du spot correspond à l'acide méta-hydroxybenzoïque). 2<sup>ème</sup> ligne: Acide succinique; phénylacétylglutamine (juste au-dessous du spot hippurique). 3<sup>ème</sup> ligne: Acide  $\alpha$ -cétoglutarique. 4<sup>ème</sup> ligne: Acide malique; acide pyrrolidonecarboxylique. 5<sup>ème</sup> ligne: Acide citrique (en traînée). *B*, 1<sup>ère</sup> ligne: Apparition d'un petit spot d'acide aconitique; augmentation considérable de l'acide fumarique. 2<sup>ème</sup> ligne: Augmentation importante de l'acide succinique. 3<sup>ème</sup> et 4<sup>ème</sup> lignes: Le spot de l'acide  $\alpha$ -cétoglutarique est très augmenté; sa queue se place entre l'acide malique également augmenté et l'acide pyrrolidonecarboxylique. 5<sup>ème</sup> ligne: Légère augmentation du spot citrique. Les autres spots ne sont pas sensiblement modifiés par rapport à *A*.





ou 0.16 mg. pour 100 cc. (36), ou encore 0.05 à 0.11 mg. pour 100 cc. (moyenne 0.08 mg. %) (47), ou enfin 0.13 mg. pour 100 cc., avec comme valeurs extrêmes 0.05 et 0.27 (12). Les résultats obtenus par une technique enzymatique d'une grande spécificité sont voisins de ceux obtenus par chromatographie, soit en moyenne 0.17 mg. pour 100 cc., chez 35 sujets normaux (152). Le rapport molaire acide  $\alpha$ -cétoglutarique/acide citrique est de  $1/9.3 \pm 14\%$  (47), dans le sang total.

Contrairement aux acides citrique et  $\alpha$ -cétoglutarique, les autres acides du cycle tricarboxylique n'ont été déterminés que de façon exceptionnelle dans le sang; certains même n'y ont pas été encore décelés.

L'*acide succinique* a pu être dosé dans le sérum humain par Thunberg (184) grâce à l'utilisation de la succinodéshydrogénase. Sa concentration physiologique est, selon cet auteur, de 0.6 à 0.7 mg. pour 100 cc. Krebs (73) trouve des chiffres voisins (0.5 mg. pour 100 cc.).

L'acides succinique sanguin a été dosé également chez le lapin, où sa concentration est de 0.42 mg. pour 100 cc. (43), tandis que cette concentration serait inférieure à 0.1 mg. pour 100 cc., dans le sang de rat (44bis).

L'*acide malique* a été dosé dans le plasma humain à l'aide d'une technique fluorométrique très sensible (62); sa concentration y est de 0.1 à 0.9 mg. pour 100 cc.

Les autres acides du cycle tricarboxylique n'ont pas été décelés, à notre connaissance, dans le sang humain. Grâce à une technique chromatographique sur colonne de silice, Frohman, Orten et Smith (44bis) ont pu montrer que dans le sang de rat la concentration des acides isocitrique et aconitique est inférieure à 0.1 mg. pour 100 cc., celle de l'acide fumarique inférieure à 0.3 mg. pour 100 cc. (93).

Nous avons réalisé une centaine de chromatogrammes plasmatiques par une technique proche de celle décrite pour l'urine. Dix cc. de plasma sont amenés à pH 5.3 par adjonction d'acide acétique, puis déprotéinisés par la chaleur. Après centrifugation et passage du surnageant sur colonne de Dowex 2, les acides organiques sont élués et chromatographiés sur papier dans les mêmes conditions que l'urine.

Les spots rencontrés le plus fréquemment sur ces chromatogrammes de plasma humain normal correspondent aux acides citrique, succinique, lactique et pyrrolidonecarboxylique. Nous n'avons par contre jamais décelé la présence d'acide aconitique ou fumarique, ce qui montre que leur concentration ne doit pas être plus élevée dans le plasma humain que dans le sang de rat.

#### LES ACIDES DU CYCLE TRICARBOXYLIQUE DANS LES LIQUIDES BIOLOGIQUES AUTRES QUE LE SANG ET L'URINE

La présence de citrate a été démontrée dans la quasi totalité des liquides biologiques: liquide céphalo-rachidien (9, 13, 61, 95), lait (59), sueur (10),

humeur aqueuse (52), liquide prostatique (149), sperme (147), liquide amniotique (106). La salive, par contre, en serait dépourvue (80).

L'acide  $\alpha$ -cétoglutarique a été décelé également dans des liquides biologiques aussi différents que la périlymphe (5) et les exsudats pathologiques (12).

Le liquide céphalo-rachidien contient environ deux fois moins de succinate que le sérum sanguin, contrairement au citrate, dont la concentration est plus élevée dans le liquide céphalo-rachidien que dans le sérum (184).

#### MODIFICATIONS PHYSIOLOGIQUES DES ACIDES DU CYCLE TRICARBOXYLIQUE DANS LES LIQUIDES BIOLOGIQUES

##### INFLUENCE DE L'ALIMENTATION

L'élimination urinaire de citrate s'élève après chaque repas (80); l'amplitude de cette augmentation dépend du type d'aliments ingérés (99, 156). Une importante élimination urinaire de citrate persiste néanmoins chez le chien soumis à un jeûne prolongé (13).

Les influences alimentaires sont plus modérées en ce qui concerne l'élimination urinaire des acides  $\alpha$ -cétoglutarique (78) et succinique (43, 181).

Les concentrations sanguines des acides citrique et  $\alpha$ -cétoglutarique sont également modifiées par les repas (47, 80, 103).

##### INFLUENCE DE L'EXERCICE MUSCULAIRE

Un exercice musculaire violent augmente l'acide citrique sanguin et diminue la citraturie (80); il ne modifie pas l'acide  $\alpha$ -cétoglutarique sanguin.

##### VARIATIONS EN FONCTION DE L'AGE ET DU SEXE

La citraturie est beaucoup plus faible chez l'enfant que chez l'adulte (88, 91, 165); l'acide citrique est cependant présent dans l'urine dès la naissance, avant même que le nouveau-né soit alimenté (13). La citraturie est également faible chez les vieillards (151).

L'élimination urinaire de citrate est maxima chez la femme aux environs du 14<sup>ème</sup> jour du cycle et s'abaisse fortement au moment des règles (158). Les hormones mâles (158), la cortisone (131) et l'insuline (103) agissent également sur les concentrations de citrate dans les liquides biologiques.

#### ROLE PHYSIOLOGIQUE DES ACIDES DU CYCLE TRICARBOXYLIQUE DANS LES LIQUIDES BIOLOGIQUES

##### ROLE METABOLIQUE ET ENERGETIQUE

Les acides du cycle citrique sont des composés jouant un rôle primordial en tant que donneurs d'énergie (75) et comme fournisseurs de métabolites essentiels dans de nombreuses réactions. Dans le sang il pourrait s'agir de produits en excès provenant d'un centre métabolique et destinés à être simplement transportés dans un autre organe pour y jouer un rôle analogue; dans l'urine il s'agit évidemment de produits éliminés représentant une perte énergétique pour l'organisme.

#### RELATIONS AVEC LE CALCIUM

Le citrate formant avec le calcium un complexe soluble non dissocié citrate-calcium, la concentration du citrate d'un liquide biologique influence la solubilité et l'ionisation du calcium contenu dans ce liquide (57, 92).

Une forte augmentation du citrate sanguin peut ainsi déterminer une chute du calcium ionisé d'une amplitude telle qu'elle provoque des accidents graves et même mortels. Une telle élévation considérable de la citratémie peut survenir au cours d'injections intraveineuses d'acide citrique ou de citrate de sodium (71, 97, 145) ou lors de transfusions rapides et massives de sang citraté (4, 18, 98, 195).

Le citrate urinaire apparaît pour sa part comme l'un des facteurs essentiels de stabilité du calcium urinaire, la formation d'un complexe soluble citrate-calcium contribuant à maintenir le calcium urinaire en solution (51, 57, 58, 59, 125, 159).

#### RELATIONS AVEC L'EQUILIBRE ACIDO-BASIQUE

L'élimination urinaire de citrate diminue après administration d'agents déterminant une acidose, alors qu'elle augmente au contraire, après administration d'alcalins (41, 125). L'accroissement de la citraturie après apport d'alcalins permettrait à l'organisme d'éliminer l'excès de cations sans faire appel aux anions fixes, le citrate formant ainsi l'un des systèmes tampons essentiels de l'urine. Le citrate jouerait donc, en ce qui concerne l'économie d'anions fixes, un rôle analogue à celui de l'ammoniurie en ce qui concerne les bases fixes (125). Lors de l'administration de doses fortes de bicarbonate de sodium au chien ou au rat, la citraturie peut atteindre 100 fois celle des animaux témoins (156). Il n'y a cependant pas de parallélisme constant entre citraturie et pH urinaire (13, 80). Ces modifications de la citraturie ne sont pas toujours accompagnées de modifications parallèles du citrate sanguin (94, 127).

### EXPLORATION DU CYCLE TRICARBOXYLIQUE A L'AIDE DES EPREUVES DE CHARGE

#### INFLUENCE DES CHARGES SUR L'ELIMINATION URINAIRE DES ACIDES DU CYCLE

Orten et Smith (122) ont étudié l'influence sur la citraturie du chien de l'administration par voie veineuse de quantités équivalentes de sels de sodium de divers acides. Ils classent ainsi les sels testés en 4 catégories selon l'importance des modifications de la citraturie déterminées par leur administration. Le groupe déterminant l'élévation la plus importante de la citraturie est formé des sels suivants: succinate, fumarate, dl-malate, malonate et maléate; tous les acides du cycle tricarboxylique testés par ces auteurs font partie de ce groupe, qui comporte de plus deux inhibiteurs enzymatiques, malonate et maléate.

Simola et Krusius (78, 162) ont décelé de même chez le rat une élévation

notable du citrate et de l' $\alpha$ -cétoglutarate urinaires lors de charges perorales en divers sels d'acides du cycle tricarboxylique. Des expériences analogues ont été réalisées également par Krebs, Salvin et Johnson (71), qui ont étudié l'influence de telles charges sur l'élimination urinaire du citrate, de l' $\alpha$ -cétoglutarate et du succinate chez le lapin et le rat. Ces auteurs décèlent ainsi une élévation assez notable de l' $\alpha$ -cétoglutarate et du citrate, tandis que l'augmentation de l'élimination urinaire du succinate est plus discrète et même parfois absente. Malgré l'intérêt de cette étude, il faut remarquer que ces résultats correspondent à des expériences uniques réalisées chacune sur un seul animal. De plus, les grandes fluctuations de l'élimination de base des acides  $\alpha$ -cétoglutarique et surtout succinique (2 à 59  $\mu$ moles par 24 heures) suggèrent une certaine imprécision des techniques utilisées alors.

Ces études, pour intéressantes qu'elles soient, ne concernent que l'élimination des acides citrique et  $\alpha$ -cétoglutarique, ainsi que, dans le travail de Krebs *et al.* (71), celle de l'acide succinique. L'élimination urinaire des autres acides du cycle tricarboxylique n'a pu être étudiée, faute de techniques adéquates. Emmrich (38) affirme même que les acides fumarique et malique n'apparaissent pas dans l'urine après charge en succinate, tout en reconnaissant que ce résultat négatif peut être dû à des techniques insuffisantes. Balassa (7), de son côté, ne décèle pas d'acide malique dans l'urine après ingestion de 5 g. de succinate de sodium, mais y trouve par contre 30 mg. d'acide fumarique; Forssman (43, p. 15) conteste pourtant ce résultat, en mettant l'accent sur le manque de spécificité de la technique utilisée par Balassa (l'acide oxalique est dosé en même temps que l'acide fumarique et le chiffre trouvé par Balassa semble correspondre à celui de l'oxalurie normale). Il faut souligner de plus que ces études, à l'exception de celle de Balassa dont nous venons de parler, concernent toutes l'animal et non l'homme.

*Pour saisir l'ensemble des modifications urinaires déterminées par les charges, une technique de détermination quantitative des divers acides du cycle tricarboxylique est indispensable. C'est pour répondre à cette nécessité que nous nous sommes consacrés à la mise au point quantitative de notre technique chromatographique, dont nous avons parlé plus haut.*

La détermination quantitative d'après la surface des spots (14, 35, 138) s'est révélée inapplicable à des chromatogrammes bidimensionnels (ne permettant donc pas la chromatographie parallèle de séries de témoins) d'éluates contenant des anions minéraux (dont la présence modifie la surface des spots de certains acides organiques). De même la technique colorimétrique après révélation par un mélange bleu de thymol/glycocolle (63) ne nous a pas donné satisfaction pour nos chromatogrammes en deux dimensions.

Nous avons donc eu recours à la titration des acides extraits des spots, malgré les difficultés inhérentes à cette méthode (138). La titration est effectuée au pH-mètre, sous azote, avec de la soude 0.01 N (69, p. 109).

On constate dans ces conditions d'importantes pertes chromatographiques

lorsqu'on utilise du papier Whatman No. 1 (par exemple 35% pour 200  $\mu$ g. d'acide succinique). L'essentiel de ces pertes n'est pas due à une perte réelle de l'acide au cours de la chromatographie, mais à une neutralisation partielle par les impuretés du papier. Si on compare, en effet, les résultats obtenus d'une part par titration, d'autre part par une technique caractérisant l'anion organique et donnant des résultats identiques, que cet anion soit sous forme d'acide libre ou de sel, on constate que la titration donne toujours des résultats inférieurs. Aussi est-il possible de réduire notablement les pertes en éliminant une partie des impuretés du papier grâce à un lavage acide. Dans ces conditions, les résultats des titrations, bien qu'encore inférieurs à ceux obtenus par une technique non influencée par une neutralisation partielle, sont parfaitement utilisables pour les déterminations quantitatives. Les pertes s'avèrent, en effet, identiques pour divers chromatogrammes réalisés parallèlement et, entre certaines limites, indépendantes de la quantité d'acide chromatographiée.

Ces faits nous ont conduit à utiliser, pour les chromatogrammes destinés à être interprétés quantitativement, non le papier Whatman 1, mais le papier Schleicher et Schühl 2043 b, mgl (a) qui est lavé industriellement par les acides silicique et chlorhydrique, ou du papier Arches 302 que nous lavons avec de l'acide chlorhydrique 0.1N et qui nous a donné de meilleurs résultats que le Whatman 1 lavé dans les mêmes conditions.

En même temps que chaque lot d'urines, nous chromatographions des acides témoins ainsi que ces mêmes acides passés sur colonne de Dowex 2 dans des conditions identiques à celles des urines, ce qui nous permet d'établir les pertes chromatographiques, ainsi que les pertes survenant lors de l'élimination des substances interférentes. Tous les chromatogrammes sont faits en double. Enfin, de façon à obtenir un contrôle supplémentaire, chaque urine est chromatographiée d'une part sans adjonction de témoins, d'autre part après adjonction de 250  $\mu$ g. de chacun des acides à déterminer quantitativement.

*Cette technique permet en particulier la détermination quantitative des acides aconitique, succinique, fumarique et malique.* Elle n'est pas applicable à l'acide citrique, dont le spot est souvent mal séparé, comme il a été dit plus haut; nous dosons cet acide par la technique de Taylor (177). Enfin, étant donné les pertes importantes survenant lors de la chromatographie de l'acide  $\alpha$ -cétoglutarique à l'état d'acide libre, nous réalisons parallèlement aux chromatogrammes d'acides organiques et aux dosages d'acide citrique, des chromatogrammes des 2,4-dinitrophénylhydrazones des acides cétoniques urinaires, selon la technique quantitative de El Hawary et Thompson (36).

Avant d'envisager les résultats obtenus ainsi lors de charges réalisées chez l'homme normal et au cours de diverses affections, nous indiquerons les principaux résultats enregistrés lors de charges effectuées chez le rat. L'intérêt de ces études chez l'animal est double: d'une part, les résultats sont plus constants que chez l'homme, étant donné la stabilité du régime et la certitude de la récolte de la totalité des urines; d'autre part, il est possible parfois de mettre

en évidence des différences métaboliques importantes entre l'homme et le rat, telle que celle dont nous parlerons à propos des charges en malate.

Toutes nos expériences de charges animales ont été réalisées sur des rats mâles adultes, d'un poids d'environ 270 g., de souche Sprague-Dawley, mis à un régime semi-synthétique décrit ailleurs (108) et placés dans des cages métaboliques permettant la récolte de la totalité des urines non mêlées à des fèces.

Nous avons effectué chez ces rats une série de charges en  $\alpha$ -cétoglutarate, succinate, dl-malate, pyruvate (seul et associé au dl-malate), à raison de 200  $\mu$ moles. du sel de sodium par 100 g. de poids, administrés par voie intrapéritonéale.

Les modifications de l'élimination urinaire des acides du cycle tricarboxylique déterminées par ces charges sont représentées dans le Tableau 1.

La quantité de l'acide injecté récupérée dans l'urine des 24 heures suivant la charge est très variable selon la substance injectée. Rappelons qu'il ressort de nombreux travaux que lors de l'administration d'acide citrique (49, 80, 120, 125),  $\alpha$ -cétoglutarique (78) ou succinique (7, 11, 89, 190) par voie orale, l'essentiel de l'acide administré est métabolisé rapidement sans être éliminé

**Tableau 1.** ELIMINATION URINAIRE DES PRINCIPAUX ACIDES DU CYCLE TRICARBOXYLIQUE CHEZ LE RAT NORMAL AU COURS DE DIVERSES CHARGES

Charge	Elimination urinaire			
	Oxalate	$\alpha$ -cétoglutarate	Succinate	Malate
	mg./100 cc.	mg./100 cc.	mg./100 cc.	mg./100 cc.
Sans charge	7.2 $\pm$ 1.9 (14)	4.0 $\pm$ 0.8 (14)	-	-
$\alpha$ -Cétoglutarate	52.2 $\pm$ 8.0 ( 5)	88.6 $\pm$ 27.4 ( 5)	21.2 $\pm$ 5.5 ( 5)	22.1 $\pm$ 1.4 ( 4)
Succinate	41.5 $\pm$ 1.8 (11)	38.1 $\pm$ 6.0 ( 8)	61.7 $\pm$ 10.6 ( 7)	25.9 $\pm$ 8.1 ( 8)
dl-Malate	48.8 $\pm$ 4.1 (17)	24.3 $\pm$ 4.0 ( 6)	24.4 $\pm$ 6.6 (13)	168.0 $\pm$ 18.8 (14)
Pyruvate	8.0 $\pm$ 4.5 ( 8)	6.1 $\pm$ 1.9 ( 8)	-	-
dl-Malate + pyruvate	79.3 $\pm$ 13.6 ( 9)	38.6 $\pm$ 5.7 ( 8)	39.5 $\pm$ 8.2 ( 6)	168.3 $\pm$ 34.4 ( 6)

Rats mâles Sprague-Dawley, d'un poids d'environ 270 Gm., mis à un régime semi-synthétique (109).

Toutes les charges ont été faites par voie intrapéritonéale à raison de 200  $\mu$ moles du sel de sodium de l'acide considéré par 100 Gm. de poids.

Les chiffres représentent l'élimination urinaire dans l'urine des 24 heures suivant la charge. Ils sont exprimés en  $\mu$ moles par 24 heures et représentent la moyenne des résultats

$\pm$  l'intervalle de confiance au risque de 5% pour la moyenne observée, soit  $\frac{2s}{\sqrt{N}}$  Le nombre d'expériences est indiqué dans chaque cas entre parenthèses.

Nous n'avons pas fait figurer dans ce tableau l'élimination des acides aconitique et fumarique, ainsi que celle des acides succinique et malique sans charge et après charge en pyruvate, les chiffres obtenus étant souvent à la limite de sensibilité de la technique.

Source: Nordmann et al. (114).

par voie urinaire, tandis qu'au contraire une proportion beaucoup plus élevée de ces acides peut échapper au catabolisme et être retrouvée dans l'urine lors des charges par voie parentérale (43, 71, 94, 122, 145, 183).

Chez nos rats, la récupération urinaire moyenne de l'acide injecté est de  $17.0 \pm 4.4\%$  de la quantité injectée lors des charges en  $\alpha$ -cétoglutarate (moyenne de 5 rats), de  $11.6 \pm 2.4\%$  lors des charges en succinate (moyenne de 7 rats) enfin de  $31.5 \pm 4.2\%$  lors des charges en *dl*-malate (moyenne de 14 rats) (le second chiffre représentant dans chaque cas l'intervalle de confiance

au risque de 5% pour la moyenne observée, soit  $\frac{2s}{\sqrt{N}}$ .

Dans le cas des charges en *dl*-malate, il faut signaler que la totalité de l'isomère *d*- injecté n'est pas éliminée dans l'urine, puisqu'après injection de *dl*-malate nous retrouvons dans l'urine moins de la moitié de la quantité administrée. Une partie de l'isomère *d*- pourrait être éliminée par une voie différente de l'excrétion urinaire, à moins qu'elle ne puisse être métabolisée sous l'influence d'une racémase.

L'élimination de citrate n'est pas différente statistiquement (au risque de 5%) après charge en succinate, ou *dl*-malate. Par contre, cette élimination est légèrement plus élevée lors des charges en  $\alpha$ -cétoglutarate que lors de celles en succinate, la différence étant statistiquement significative.

L'élimination urinaire d' $\alpha$ -cétoglutarate est significativement plus élevée après charge en succinate que lors de celles en *dl*-malate. Cette forte élimination d' $\alpha$ -cétoglutarate après charge en succinate semble en rapport avec l'équilibre stoechiométrique succinate  $\longleftrightarrow$   $\alpha$ -cétoglutarate.

Le rapport citrate/ $\alpha$ -cétoglutarate est inférieur à 2 dans l'urine du rat normal en l'absence de charge. Il est ainsi bien différent de celui trouvé dans l'urine humaine, où il est de 15.8, ainsi que nous le verrons plus loin. Ce rapport augmente légèrement lors des charges en *dl*-malate, indiquant que le citrate est le premier corps formé à partir du malate injecté, l' $\alpha$ -cétoglutarate ne se formant qu'ultérieurement à partir du citrate. Le malate se métaboliserait donc essentiellement chez le rat par la voie du cycle tricarboxylique. Nous verrons que nos constatations sont bien différentes chez l'homme où, au contraire, le rapport citrate/ $\alpha$ -cétoglutarate s'abaisse fortement au cours de ces charges.

Lors de l'injection simultanée de *dl*-malate + pyruvate, l'augmentation de l'élimination urinaire de citrate,  $\alpha$ -cétoglutarate et succinate est beaucoup plus forte que lors des charges en *dl*-malate seul et ceci bien que l'injection de pyruvate seul ait peu d'influence sur l'élimination de ces acides. Ce fait peut être interprété comme le témoin d'un manque relatif de pyruvate lors des charges en malate seul et d'un manque d'oxaloacétate lors des charges en pyruvate seul. La formation de citrate à partir de l'oxaloacétate résultant du malate injecté nécessite la condensation avec du pyruvate, qui peut être soit du



pyruvate préexistant, soit du pyruvate provenant lui-même de la décarboxylation d'une partie du malate injecté. Lors de l'injection de malate sans administration parallèle de pyruvate, le taux relativement faible de formation de citrate (par rapport à celui observé lors de l'injection simultanée de pyruvate + malate) peut s'expliquer par un manque de pyruvate disponible et probablement aussi par l'utilisation partielle du malate pour former du pyruvate, ce qui a pour conséquence la diminution de l'oxaloacétate disponible pour la formation de citrate.

Ayant ainsi établi les modifications des acides du cycle tricarboxylique dans l'urine à la suite d'un certain nombre de charges chez le rat; nous avons effectué également des charges chez l'homme afin d'en comparer les résultats.

Nous n'avons réalisé jusqu'ici que des charges en succinate et en malate. Celles-ci ont été faites en perfusant par voie veineuse 1 mMole de succinate ou dl-malate de sodium par Kg. de poids, le volume total perfusé étant de 250 cc. (durée de la perfusion: 30 minutes). Le nombre de résultats acquis ne permet pas encore une étude statistique.

Les perfusions de succinate sont habituellement parfaitement tolérées; dans un certain nombre de cas, cependant, on note l'apparition de bouffées vasomotrices transitoires avec sensation de chaleur et rougeur, localisées surtout à la face, et parfois prurit. Ces troubles ont toujours été très fugaces et nous n'avons jamais observé de signes alarmants. Les expériences animales ont d'ailleurs montré l'absence de toxicité du succinate à de telles doses (43, pp. 78-81; 122-188).

La tolérance des perfusions de malate que nous avons réalisées chez l'homme a toujours été parfaite, sans que nous ayons jamais noté l'apparition de petits troubles tels que ceux décrits plus haut avec le succinate. Malgré l'action dépressive du malate sur le coeur isolé de grenouille (146), nous n'avons, en particulier, jamais noté aucune action cardio-vasculaire au cours de ces perfusions.

*Lors des perfusions en succinate, on constate sur les chromatogrammes des acides organiques des urines émises à la suite de cette perfusion une augmentation des divers acides faisant partie du cycle tricarboxylique. Cette augmentation est sélective, comme en témoigne le fait que les autres acides organiques apparaissant sur les chromatogrammes ne sont pas notablement modifiés; de plus nous n'avons pas constaté une telle augmentation des acides du cycle tricarboxylique en perfusant dans les mêmes conditions une quantité équivalente de bicarbonate de sodium, ce qui démontre que cet accroissement ne peut être dû à une simple action alcalinisante des perfusions de succinate. Les modifications de ces acides apparaissent avec une particulière netteté sur les chromatogrammes de l'urine émise dès la fin de la perfusion, ainsi que sur ceux de l'urine recueillie 90 minutes plus tard. Le retour à la normale est alors progressif et se fait en 12 heures au plus. La Figure 4 (p. 469) montre, à*



titre d'exemple, les chromatogrammes de l'urine d'un même sujet avant et aussitôt après perfusion de succinate.

La Figure 5 indique les quantités des principaux acides du cycle tri-carboxylique trouvées en excès dans l'urine des 24 heures suivant une telle perfusion (par rapport à l'urine de la veille de la perfusion). Ce tableau représente la moyenne trouvée chez six sujets sains pesant de 55 à 75 Kg., la quantité de succinate perfusée étant donc de 55 à 75 mMoles. La faible augmentation de l'élimination urinaire de succinate est remarquable, puisqu'on

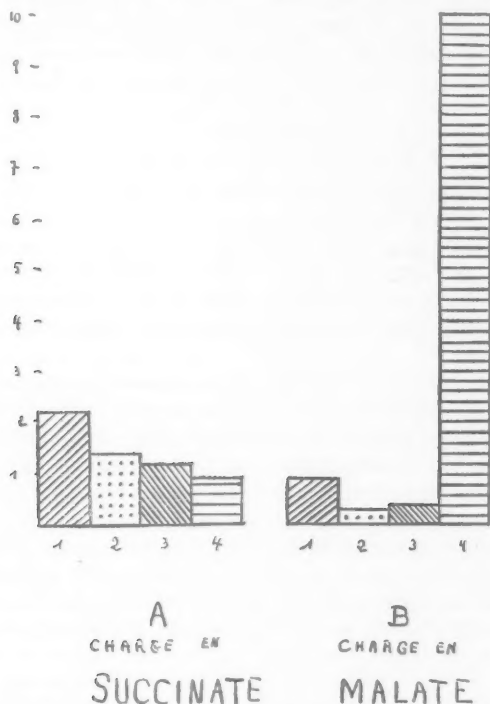


Fig. 5. Acides du Cycle Tricarboxylique Éliminés en Excès dans l'Urine des 24 Heures Suivant une Perfusion de Succinate et de Malate chez l'Homme Normal (Nordmann *et al.* [114]). A, charge en succinate. B, Charge en dl-malate. Colonnes: 1, citrate; 2, α-cétoglutarate; 3, succinate; 4, malate.

Les chiffres correspondent à l'excès d'acides éliminé dans l'urine des 24 heures suivant la perfusion par rapport à l'urine des 24 heures précédant cette perfusion; ces chiffres sont exprimés en millimoles (moyenne de 6 sujets normaux de 55 à 75 kg.). La charge a été effectuée sous forme de perfusions veineuses de 1mMole/kg. de poids de l'acide considéré, dissous dans 250 cc. d'eau distillée amené à pH 7.4 et perfusé en 30 minutes.

ne trouve en moyenne qu'un excès de 1.25 mMol, soit environ 1.66 à 2.30% de la quantité perfusée. Cette quantité apparaît beaucoup plus faible que celle retrouvée lors de nos charges en succinate chez le rat, mais il n'est pas possible de tirer argument de cette discordance en faveur d'une vitesse de métabolisme plus grande du succinate chez l'homme par rapport au rat; les doses injectées sont, en effet, deux fois plus fortes chez le rat et la voie d'introduction est différente (injection intrapéritonéale rapide au lieu de perfusion veineuse en 30 minutes). L'augmentation de l'élimination urinaire des acides citrique,  $\alpha$ -cétoglutarique et malique représente au total 4.5 mMoles; celle des acides aconitique et fumarique est discrète (les résultats obtenus jusqu'ici étant souvent à la limite inférieure de sensibilité de la technique, nous ne les avons pas indiqués dans la Fig. 5). Il apparaît donc qu'au total l'essentiel du succinate est métabolisé chez l'homme sans être éliminé dans l'urine et sans donner lieu à la formation d'acides du cycle tricarboxylique éliminés par voie urinaire. Nous discuterons plus loin de l'intérêt de la considération des rapports entre les divers acides du cycle tricarboxylique dans les échantillons d'urines recueillies à plusieurs moments après la charge.

Pour préciser si le métabolisme du malate se déroulait également par la voie du cycle tricarboxylique, nous avons réalisé des *perfusions* analogues en *dl-malate*. Les quantités des principaux acides du cycle tricarboxylique retrouvées en excès dans l'urine des 24 heures suivant une telle perfusion sont représentées dans la Fig. 5.

On voit qu'une quantité notable de malate apparaît dans les urines au cours de ces perfusions, à savoir environ 10 mMoles, sur 55 à 75 injectés, c'est-à-dire 13 à 18%. Ce chiffre est cependant inférieur à celui trouvé lors des charges en *dl-malate* chez le rat (31.5%), mais les mêmes arguments que ceux exposés à propos des charges en succinate s'appliquent au cas présent.

L'élimination des principaux autres acides du cycle tricarboxylique est modérément accrue lors de ces charges en malate, comme le montre la Fig. 5. Nous verrons plus loin que la considération des rapports entre ces différents acides dans les divers échantillons d'urine recueillis après charge en malate apporte des arguments en faveur d'un processus différent du cycle tricarboxylique.

#### ORIGINE MÉTABOLIQUE ET LIEU DE FORMATION

L'accroissement de l'élimination urinaire des divers acides faisant partie du cycle tricarboxylique à la suite de l'administration de l'un d'eux étant ainsi établie, le problème se pose de savoir dans quel organe et par quel processus ces acides sont formés.

Le fait que l'augmentation de l'élimination urinaire porte sur les divers acides du cycle et non sur le seul acide citrique permet déjà d'éliminer l'hypothèse d'une simple mobilisation de cet acide à partir des importantes

réserves de citrate osseux. L'hypothèse d'une telle mobilisation est d'ailleurs également exclue par le fait que des charges répétées en malade chez le rat ne déterminent pas de diminution significative de la teneur des os en citrate (29).

Ayant ainsi rejeté la possibilité d'une mobilisation du citrate osseux au cours des expériences de charge, il nous faut tenter de localiser dans un organe le siège du catabolisme des acides injectés.

*Un nombre relativement important de travaux plaide en faveur du siège essentiellement intra-rénal du catabolisme citrique.* Ces travaux sont basés en particulier sur l'étude de l'utilisation de cet acide par les organes isolés et perfusés ainsi que sur la considération des différences entre les concentrations artérielles et veineuses du citrate in vivo (43, 94, 163).

Trois organes ont surtout été considérés comme siège possible du catabolisme citrique: le foie, le muscle, le rein.

La vitesse d'oxydation du citrate lors du passage du sang à travers le foie est controversée. Selon Sjöström (163), le foie élimine rapidement l'acide citrique du courant sanguin. Selon Martensson (94), au contraire, le foie isolé du lapin ou du chat perfusé avec une solution de citrate n'oxyde que 1.3  $\mu$ g. de citrate par minute et par gramme de foie. Les résultats montrant une importante oxydation intrahépatique du citrate seraient en rapport avec des expériences techniquement imparfaites, entraînant une lésion hépatique.

De nombreuses expériences montrent que l'acide citrique n'est pas notablement catabolisé dans le muscle (94).

Le troisième organe qui entre essentiellement en ligne de compte, *le rein, est au contraire le siège d'un intense catabolisme citrique.* Lors de perfusions continues de citrate maintenant le taux sérique du citrate à 0.9 mg. %, le rein oxyde ainsi 32  $\mu$ g. de citrate par minute et par gramme de rein. Si une partie de l'acide citrique est éliminée par voie urinaire, l'essentiel est cependant non pas éliminé par cette voie, mais bien oxydé dans le parenchyme rénal, car *l'acide citrique perfusé continue à être consommé par le rein alors que toute formation d'urine a cessé* (94).

L'étude des différences artério-veineuses chez l'animal, en dehors de toute perfusion de citrate, confirme le rôle prépondérant du rein dans le catabolisme normal de cet acide. La teneur en citrate de la veine rénale est, en effet, inférieure de 30% chez le lapin, de 20% chez le chat à celle de l'artère rénale (94).

Ainsi donc le siège essentiel de l'oxydation du citrate in vivo est le rein. Le citrate ne pourrait pénétrer à l'intérieur du tissu hépatique ou musculaire normal, alors qu'il pénétrerait aisément à l'intérieur des cellules rénales. Les différences entre les résultats obtenus in vivo et ceux trouvés in vitro sur des homogénats ou des organites cellulaires seraient en rapport avec des phénomènes de perméabilité cellulaire.

Le siège rénal du catabolisme n'est pas le fait exclusif du citrate. Les travaux de Forssman (43) ont montré, en effet, que *le succinate est également*

*catabolisé pour l'essentiel dans le rein*, comme en témoigne en particulier l'étude comparative des différences artério-veineuses de divers organes. Alors que le foie et le muscle ne métabolisent que faiblement et lentement le succinate, le rein élimine rapidement le succinate du courant sanguin. Cette élimination n'est que partiellement en rapport avec l'excrétion urinaire, qui ne représente au maximum que 5% de la quantité administrée au cours d'expériences de charge en succinate; l'essentiel de cette élimination serait en rapport avec une oxydation intra-rénale du succinate. Le rôle prépondérant du rein apparaît, en particulier, au cours d'expériences comparatives de charges en succinate réalisées d'une part chez des lapins normaux, d'autre part chez des lapins néphrectomisés. Après injection d'une même quantité de succinate, l'élévation du succinate sanguin est, en effet, plus marquée et plus durable chez les animaux néphrectomisés que chez les animaux témoins. Par contre, la néphrectomie sans administration de succinate ne modifie que peu la concentration du succinate sanguin.

Le rein apparaissant ainsi comme le siège essentiel du catabolisme du citrate et du succinate, il faut se demander s'il en est de même pour le catabolisme de la totalité des acides du cycle tricarboxylique et si les divers acides éliminés dans l'urine lors des charges en substrats du cycle tricarboxylique sont toujours et exclusivement formés dans le rein par l'intermédiaire du cycle tricarboxylique.

Les résultats de Smith et Orten (123, 164) s'inscrivent en faveur d'une telle formation intrarénale du citrate trouvé dans l'urine après charge en malate chez le rat. Ces auteurs ont dosé le citrate urinaire, sanguin et tissulaire après charge en malate de sodium chez cet animal et comparé les résultats obtenus avec ceux d'une série de rats témoins recevant une même dose de sodium sous forme de chlorure de sodium. Ils constatent ainsi l'absence de différence significative entre les deux groupes d'animaux en ce qui concerne l'acide citrique sanguin, hépatique et musculaire. Les reins des animaux ayant reçu du malate contiennent par contre nettement plus de citrate que ceux des animaux témoins (14.9 mg. % au lieu de 6.3 mg. %). De plus en réalisant ce même type de charges chez les rats néphrectomisés, il n'apparaît pas de différence significative dans les taux de citrate sanguin, musculaire et hépatique entre les rats recevant du malate et ceux recevant du chlorure de sodium, ce qui démontre le rôle fondamental du rein dans la formation de citrate à partir de malate chez le rat.

Le fait que, chez le rat, le taux sanguin de citrate s'élève à peine lors des charges en succinate, fumarate et malate, bien que des quantités importantes de citrate soient éliminées au même moment dans l'urine, représente un argument supplémentaire en faveur de la formation intrarénale de ce citrate (164).

*Il faut cependant se demander si la totalité des acides éliminés dans l'urine au cours des diverses charges est ainsi formée dans le rein par l'intermédiaire du cycle tricarboxylique, aussi bien chez l'homme que chez l'animal.*

*Les charges en malate que nous avons réalisées chez l'homme nous permettent d'affirmer qu'une telle généralisation serait erronée.*

*Si nous comparons, en effet, le rapport citrate/ $\alpha$ -cétoglutarate dans l'urine des 24 heures précédant une telle charge en malate à ce même rapport dans l'urine des 24 heures suivant cette charge, nous constatons que ce rapport augmente légèrement chez le rat. Cette augmentation paraît confirmer l'hypothèse d'une formation de ces deux acides (à partir du malate injecté) par l'intermédiaire du cycle tricarboxylique, le citrate étant formé avant l' $\alpha$ -cétoglutarate.*

*Chez l'homme, au contraire, ce rapport urinaire s'abaisse de 15.8 à 6.8, abaissement qui démontre l'élimination d'un excès relatif d' $\alpha$ -cétoglutarate par rapport au citrate.*

Pour mieux saisir ce processus, nous avons étudié l'élimination des divers acides du cycle tricarboxylique non seulement dans un échantillon de l'urine des 24 heures suivant la perfusion de malate, mais également dans des échantillons d'urine recueillis aussitôt après la fin de la perfusion, ainsi que 90 minutes et 12 heures après cette fin. Les résultats en sont consignés dans la Figure 6.

*On constate ainsi que l'élimination d'acide  $\alpha$ -cétoglutarique atteint un maximum dans l'échantillon d'urine émis dès la fin de la perfusion. Celle de l'acide citrique, au contraire n'atteint ce maximum que dans l'échantillon d'urine recueilli 90 minutes plus tard. Ce décalage entre les pics respectifs d'élimination de l' $\alpha$ -cétoglutarate et du citrate s'oppose à la conception d'une formation de l' $\alpha$ -cétoglutarate à partir du citrate, telle qu'elle devrait survenir dans l'hypothèse d'un métabolisme du malate injecté par la seule voie du cycle tricarboxylique. La considération des rapports entre les divers acides éliminés dans ces échantillons d'urine recueillis à différents moments après la perfusion de malate (Tableau 2) met bien en évidence cet accroissement prépondérant de l' $\alpha$ -cétoglutarate, puisque le rapport citrate/ $\alpha$ -cétoglutarate s'abaisse de 15.8 avant perfusion à 1.55 aussitôt après, pour remonter ensuite progressivement.*

Pour expliquer l'augmentation immédiate et prépondérante de l' $\alpha$ -cétoglutarate, il faut faire intervenir un processus différent du cycle tricarboxylique. L'explication la plus probable est la formation d' $\alpha$ -cétoglutarate par transamination entre l'oxaloacétate résultant du malate injecté et le glutamate (donnant lieu à la formation d' $\alpha$ -cétoglutarate et d'aspartate). Nous nous proposons de doser les acides glutamique et aspartique après charge en malate pour confirmer cette hypothèse.

*Il est probable qu'une telle transamination siège non dans le rein, mais dans des organes particulièrement riches en transaminase glutamique-oxaloacétique, tels que le foie et surtout le coeur (198). L'étude des modifications sanguines du citrate et de l' $\alpha$ -cétoglutarate au cours de ces charges en malate nous a révélé, en effet, une augmentation très importante de l' $\alpha$ -cétoglutarate, tandis que le citrate n'est que modérément augmenté. Le rapport molaire citrate/ $\alpha$ -*

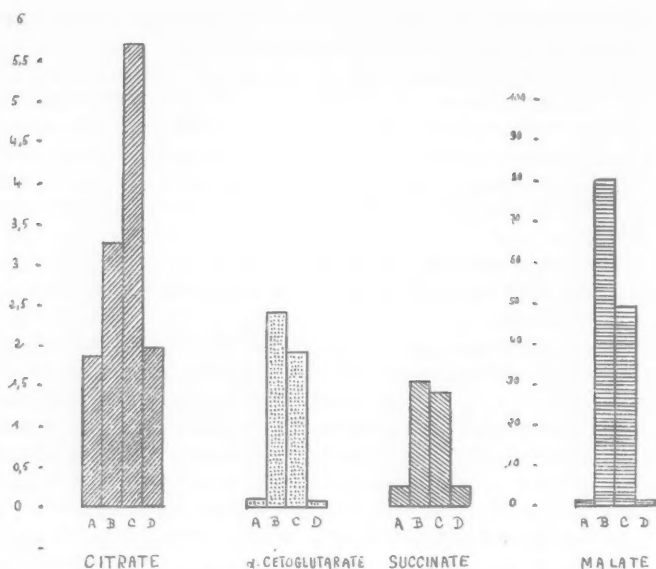


Fig. 6. Modifications des Principaux Acides du Cycle Tricarboxylique dans l'Urine Humaine Normale sous l'Influence d'une Perfusion Veineuse de 1 mMole./kg. de *dl*-Malate (Nordmann *et al.* [114]). Conditions expérimentales identiques à celles de Fig. 5. Tous les résultats sont exprimés en mmoles d'acide pour 1 mg. de créatinine urinaire. A, avant perfusion. B, aussitôt après la perfusion. C, 90 minutes après la fin de la perfusion. D, 12 heures après la fin de la perfusion.

Tableau 2. PRINCIPAUX RAPPORTS DES ACIDES DU CYCLE TRICARBOXYLIQUE DANS L'URINE HUMAINE NORMALE APRÈS CHARGE EN *dl*-MALATE

	Malate : citrate (mg./100 cc.)	Citrate : α-céto- glutarate (mg./100 cc.)	α-céto-glutarate : succinate (mg./100 cc.)	Succinate : malate (mg./100 cc.)
Avant perfusion	< 0.16	15.8	> 0.35	—
Aussitôt après	21.2	1.55	1.59	0.02
90 Minutes après	8.4	3.0	1.31	0.03
12 heures après	< 0.16	13.2	> 0.41	—

Conditions expérimentales identiques à celles de la Figure 5.

cétoglutarate s'abaisse ainsi dans le plasma de 16.5 avant perfusion de malate à 1.75 à la fin de la perfusion, pour remonter à 2.75 quinze minutes après cette fin. L'augmentation importante de l'α-cétoglutarate plasmatique représente un argument important en faveur de sa formation extra-rénale.

Nous pensons avoir ainsi mis en évidence une différence métabolique notable entre l'homme et le rat lors des charges en malate, l'oxaloacétate résultant du malate étant métabolisé initialement en citrate par la voie du cycle tricarboxylique chez le rat, étant au contraire essentiellement transaminé en  $\alpha$ -céto-glutarate chez l'homme (Fig. 7).

On peut rapprocher cette différence métabolique entre l'homme et le rat de celle mise en évidence par Simola et Krusius (162; 78, pp. 142-145) lors des charges en pyruvate. Ces auteurs ont constaté, en effet, que de telles charges en pyruvate déterminent chez le rat un accroissement important de l'élimination urinaire des acides citrique et  $\alpha$ -céto-glutarique, tandis que chez l'homme et le lapin l'augmentation de l'élimination urinaire est beaucoup plus marquée en ce qui concerne l' $\alpha$ -céto-glutarate que le citrate.

Une telle différence pourrait également être en rapport avec un processus de transamination, le pyruvate se transaminant chez l'homme et le lapin avec le glutamate pour donner de l' $\alpha$ -céto-glutarate et de l'alanine. Il faut signaler cependant que Seitz *et al.* (152) ont constaté récemment la stabilité de la pyruvicémie chez l'homme lors des charges en glutamate, aussi bien que lors des charges en alanine. Ils en tirent argument pour affirmer que la réaction de transamination entre acides pyruvique et glutamique ne se fait pas de façon notable chez l'homme normal, contrairement à celle entre acides oxaloacétique et glutamique.

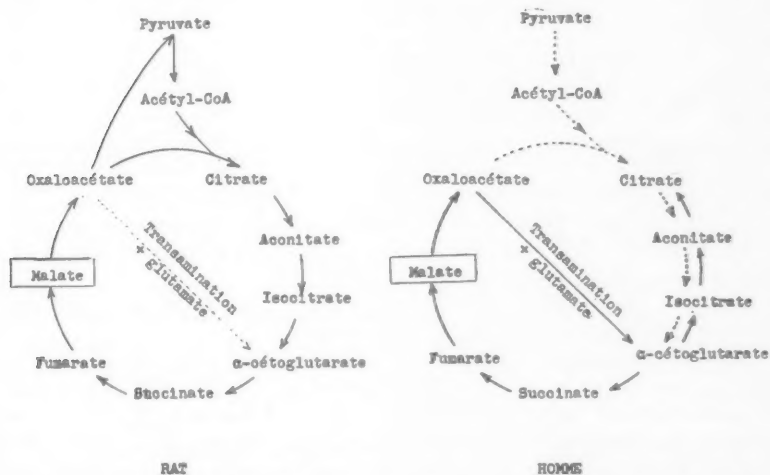


Fig. 7. Schéma hypothétique expliquant les divergences des résultats des charges en malate chez le rat et chez l'homme.

## MODIFICATIONS PATHOLOGIQUES DU CYCLE TRICARBOXYLIQUE

LES ACIDES DU CYCLE TRICARBOXYLIQUE DANS LES LIQUIDES  
BIOLOGIQUES AU COURS DE L'INSUFFISANCE HEPATIQUE

L'augmentation de l'acide citrique sanguin au cours des affections hépatiques a été décrite pour la première fois par Thunberg (183). Par la suite, différents travaux ont mis l'accent sur l'intérêt du dosage de l'acide citrique sanguin pour le diagnostic des maladies hépatiques, et, en particulier, pour le diagnostic différentiel entre ictère par hépatite et ictère par rétention (15, 47, 52, 77, 83, 85, 118, 119, 163, 194).

Dans une étude portant sur 300 malades atteints d'affections hépatiques, l'acide citrique sanguin fut ainsi trouvé supérieur à 2.4 mg. pour 100 cc. dans 79% des cas d'hépatite aiguë, dans 81% des cas d'hépatite chronique et de cirrhoses, tandis que son taux était habituellement normal dans l'ictère par lithiase cholédocienne, les affections vésiculaires, les cancers biliaires et pancréatiques; ce taux était enfin variable au cours des tumeurs hépatiques (163). Une autre étude importante (53) fait état de 81 cas d'ictère catarrhal; dans 71 de ces cas, la citratémie était élevée (avec un chiffre maximum de 10.5 mg. pour 100 cc.), tandis que les taux trouvés au cours des ictères par rétention lithiasique étaient toujours inférieurs à 2.8 mg. pour 100 cc., chiffre considéré dans cette étude comme limite supérieure de la normale.

L'augmentation de l'acide citrique au cours des hépatites apparaît souvent précocément et ne dure habituellement que peu de temps (85); une hypercitratémié importante et durable indiquerait généralement une évolution cirrhogène (119).

Il n'y a cependant pas de corrélation entre le degré d'augmentation de la citratémie et celui de l'atteinte cellulaire hépatique estimée par ponction-biopsie (194); un malade ayant ainsi une nécrose extensive des cellules parenchymateuses, qui entraîna la mort, avait un taux d'acide citrique sanguin normal (194). Il ne paraît donc pas possible d'interpréter les modifications de la citratémie au cours des hépatites comme un test fonctionnel hépatique (97).

Notons enfin que l'élévation de la citratémie au cours des cirrhoses est rarement importante; c'est ainsi que des taux supérieurs à 4 mg. pour 100 cc. ne furent rencontrés que dans 6 cas sur 55 (194).

L'acide  $\alpha$ -cétoglutarique sanguin est également augmenté au cours des affections hépatiques (12, 22, 47, 104, 154, 167).

Seligson, McCormick et Sborov (154) trouvent ainsi des taux de  $0.35 \pm 0.17$  mg. pour 100 cc. au cours des hépatites virales, au lieu de  $0.19 \pm 0.02$  chez les sujets normaux. D'autres études font cependant état de taux plus faibles au cours des hépatites (en moyenne 0.225 mg. pour 100 cc) (167), tandis que l'augmentation de l'acide  $\alpha$ -cétoglutarique sanguin serait plus importante au cours des cirrhoses (0.365 mg. pour 100 cc.) et serait en corrélation avec le degré de l'atteinte cellulaire hépatique.



Enfin cet acide a été trouvé augmenté dans un cas de coma hépatique (22), la taux y étant environ double de la normale, alors qu'au contraire un taux inférieur à la normale a été rapporté dans un autre cas de coma hépatique, l'acide  $\alpha$ -cétoglutarique s'abaissant lors de l'installation du coma (25).

L'augmentation des acides citrique et  $\alpha$ -cétoglutarique sanguins au cours de la plupart des affections hépatiques étant ainsi établie, la question se pose de savoir si une telle augmentation ne porte que sur ces deux seuls acides ou, au contraire, s'il n'y a pas au cours de l'insuffisance hépatique une augmentation de tous les acides du cycle tricarboxylique dans le sang, et éventuellement dans les autres liquides biologiques, en particulier l'urine (139).

En raison des difficultés techniques de dosage, que nous avons signalées plus haut, aucun des autres acides du cycle tricarboxylique n'avait été dosé, à notre connaissance, dans le sang des malades atteints d'affections hépatiques.

Les chromatogrammes des acides organiques plasmatiques que nous avons réalisés au cours de comas hépatiques suggèrent l'existence d'une augmentation des taux sanguins des acides succinique et malique, mais le nombre des cas étudiés jusqu'ici est trop restreint pour que nous puissions en tirer une conclusion définitive.

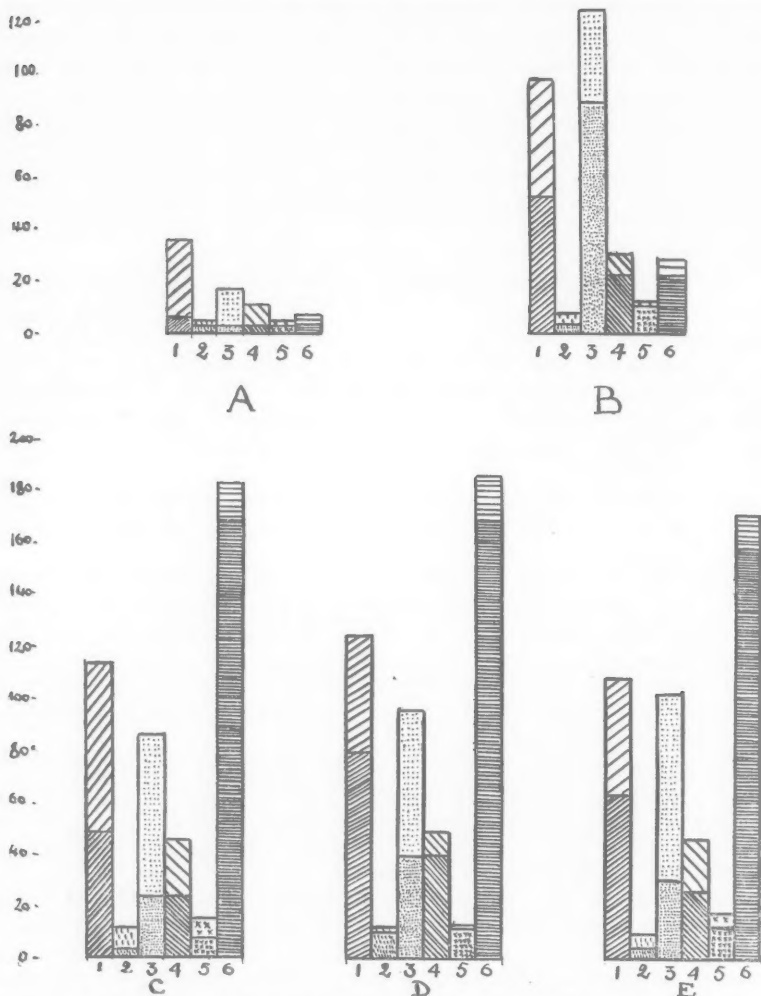
On possède de rares documents sur les variations de l'élimination urinaire des acides du cycle tricarboxylique chez les malades présentant une insuffisance hépatique.

L'élimination urinaire des acides citrique et  $\alpha$ -cétoglutarique a été étudiée chez 5 de ces malades par Krusius et Vesa (77), qui ont trouvé une élimination normale d'acide citrique, tandis que celle de l'acide  $\alpha$ -cétoglutarique paraissait dans l'ensemble élevée (un cas d'atrophie hépatique s'accompagnait cependant d'une élimination normale d'acide  $\alpha$ -cétoglutarique). Ainsi que le soulignent les auteurs eux-mêmes, il n'est pas possible de tirer de conclusion significative d'un nombre aussi restreint de cas.

Etudiant l'élimination urinaire de ces deux acides chez 15 malades atteints d'ictère catarrhal et de cirrhose éthylique, nous avons nous-mêmes trouvé une élimination de citrate à la limite supérieure de la normale, tandis que celle de l' $\alpha$ -cétoglutarate était augmentée (en moyenne 28.2 mg. avec des chiffres extrêmes de 18.2 et 42.3 mg. par 24 heures).

Selon Emmrich (39), l'acide succinique est également augmenté dans l'urine des malades atteints d'affections hépatiques. Alors que la technique utilisée par cet auteur ne lui permet pas de déterminer l'acide succinique dans l'urine normale, il trouve une élimination de 22 mg. d'acide succinique par jour dans un cas d'ictère catarrhal prolongé et de 188 mg. par jour dans un cas de foie gras tuberculeux.

Les chromatogrammes urinaires que nous avons réalisés confirment cette augmentation de l'élimination urinaire de succinate. Nous effectuons à l'heure actuelle l'étude quantitative statistique de cette augmentation, ainsi que celle des modifications portant sur les autres acides du cycle tricarboxylique. Il



**Fig. 8.** Elimination Urinaire des Acides du Cycle Tricarboxylique au Cours de Diverses Charges chez le Rat Intoxiqué par le Tétrachlorure de Carbone (Nordmann *et al.* [114]). *A*, Sans charge. *B*, Charge en  $\alpha$ -cétoglutarate. *C*, Charge en *dl*-malate. *D*, Charge en pyruvate + *dl*-malate. *E*, Charge en glutamate + *dl*-malate. Colonnes: 1, citrate; 2, aconitate; 3,  $\alpha$ -cétoglutarate; 4, succinate; 5, fumarate; 6, malate.

Les chiffres représentent l'élimination urinaire en  $\mu$  moles, dans l'urine des 24 heures suivant la charge (moyenne de 5 rats). (Se continue à la page 489)

nous faut signaler que les chromatogrammes des acides organiques urinaires de malades atteints de coma hépatique montrent la présence de nombreux spots, non encore identifiés, que l'on ne rencontre pas dans l'urine humaine normale.

Si l'on dispose ainsi d'un certain nombre de documents sur les modifications sanguines et urinaires des acides du cycle tricarboxylique chez les malades atteints d'affections hépatiques, ces documents sont encore trop rares et trop parcellaires pour localiser avec précision un trouble éventuel du cycle tricarboxylique. Une *étude expérimentale* peut être d'un grand secours, car elle permet de comparer des cas plus homogènes et d'éviter la gêne apportée par les thérapeutiques (glutamate, cortisone, etc.) dans les cas humains sévères.

Un fait essentiel a été établi ainsi par Boothby et Adams (13). Ces auteurs ont montré, en effet, que l'hépatectomie est suivie chez le chien d'une forte augmentation de l'acide citrique urinaire. L'acide citrique disparaît pratiquement de l'urine au cours des 4 heures suivant immédiatement l'opération, mais il augmente ensuite pour atteindre des taux dépassant habituellement de beaucoup les valeurs normales. Un des chiens hépatectomisés élimina ainsi 44 mg. de citrate pour 100 cc. d'urine, soit 118 mg. en 1 heure, le total éliminé en 13 heures atteignant 1.13 g.

Nous-mêmes avons réalisé chez le rat des *intoxications par le tétrachlorure de carbone* et étudié leur influence sur l'élimination urinaire des acides du cycle tricarboxylique.

Nous avons pu mettre ainsi en évidence une augmentation de tous les acides stables du cycle tricarboxylique (Fig. 8).<sup>2</sup>

Il faut signaler que des dosages quotidiens nous ont révélé la quasi-impossibilité d'obtenir des résultats constants en ce qui concerne cette élimination urinaire des acides du cycle tricarboxylique chez les rats tétrachlorurés. Cette variabilité est en rapport avec la susceptibilité individuelle des animaux et surtout avec la dose de tétrachlorure administrée. La voie d'introduction du toxique ne semble pas jouer un rôle prépondérant; nous avons comparé, en effet, l'action de trois modes d'administration du tétrachlorure sur les acides

<sup>2</sup>Dans un travail paru depuis la rédaction de cet article, L. A. Barnes, H. Moeksi et P. György (*J. Biol. Chem.* 221, 93, 1956) ont trouvé au contraire une diminution de l'acide succinique urinaire au cours d'une variété de néerose hépatique diététique chez le rat, alors que l'excrétion d'acide méthylmalonique (dont le Rf est voisin de celui de l'acide succinique) est fortement accrue. Il est probable que la différence entre les conclusions de Barnes et coll. et les nôtres tient au fait que les deux études ne portent pas sur le même type de lésion hépatique. N'ayant pas disposé d'acide méthylmalonique témoin, nous ne pouvons cependant exclure la possibilité d'une confusion entre les spots des acides méthylmalonique et succinique dans nos expériences.

**Fig. 8 (cont.)** Les charges ont été effectuées dans des conditions identiques à celles du Tableau 1. Le tétrachlorure de carbone a été administré tous les deux jours à la dose de 0.2 cc. par 100 g. de poids en injections sous-cutanées. Trois injections ont été faites avant les charges.

Les zones plus sombres des colonnes correspondent aux résultats obtenus parallèlement sur les rats normaux.

du cycle tricarboxylique urinaires: l'injection sous-cutanée de tétrachlorure pur tous les deux jours, l'injection sous-cutanée au même rythme de tétrachlorure dans l'huile d'olive, enfin l'inhalation quotidienne de vapeurs de tétrachlorure de carbone; aucune de ces méthodes ne s'est avérée donner des résultats nettement plus constants que les autres. Nous avons adopté finalement la technique consistant à injecter tous les deux jours par voie sous cutanée 0.2 cc. de tétrachlorure de carbone pur par 100 g. de poids.

La dose totale administrée depuis le début de l'intoxication joue un rôle prépondérant. Alors que l'augmentation de l'élimination urinaire des acides du cycle tricarboxylique est très importante lors des premières injections de tétrachlorure, on constate une baisse de cette élimination jusqu'à des valeurs inférieures à celles des animaux témoins chez les rats ayant reçu un nombre élevé d'injections de tétrachlorure de carbone. Il est probable que cette baisse secondaire est due à l'association d'une atteinte rénale à la lésion hépatique, les contrôles anatomopathologiques nous ayant toujours montré d'importantes lésions rénales chez les rats ayant reçu plus de 5 à 8 injections de tétrachlorure. Il est donc important de préciser que tous les résultats dont nous faisons état correspondent à des rats ayant reçu 3 injections de tétrachlorure.

*Le problème essentiel à résoudre est de trouver la cause de l'augmentation des acides du cycle tricarboxylique sanguins et urinaires au cours de l'insuffisance hépatique humaine et expérimentale.*

Plusieurs auteurs se sont attachés en particulier à déterminer le mécanisme de l'hypercitrémie, dont nous avons dit la fréquence en cas d'atteinte hépatique.

*Cette hypercitrémie apparaît liée à une diminution de la vitesse du catabolisme citrique, comme en témoigne le fait que l'augmentation de la citratémie est beaucoup plus importante et durable lors de l'administration de citrate à des sujets présentant une atteinte hépatique que lors de la même administration à des sujets normaux (97, 163, 183). C'est ainsi que l'acide citrique sanguin ne s'élève que très transitoirement chez les sujets normaux et chez les malades atteints d'ictère par rétention après absorption de 2 g. de citrate, alors que cette augmentation est durable, évidente encore au bout de 2 heures, en cas d'ictère par hépatite (163). Malgré l'intérêt de cette constatation, qui pourrait dans une certaine mesure contribuer au diagnostic de l'insuffisance hépatique, Martius (97) déconseille la pratique de charges en citrate de sodium chez les malades atteints d'affections hépatiques, le risque d'accidents en rapport avec l'influence du citrate sur le calcium ionisé étant particulièrement important chez ces malades du fait de la longue persistance de l'hypercitrémie. Rappelons également que les accidents d' "intoxication citrique" relatés lors de transfusions massives et rapides de sang citraté surviennent plus volontiers en cas d'insuffisance hépatique (18).*

Cette diminution du catabolisme citrique pourrait être attribuée aisément

à l'atteinte hépatique si l'on admet que le foie normal catabolise fortement le citrate (163).

La plupart des travaux ayant montré cependant que le catabolisme citrique normal a lieu essentiellement dans le rein et non dans le foie, il faut admettre *l'intervention d'un mécanisme indirect par l'intermédiaire duquel la lésion hépatique détermine une diminution du catabolisme citrique intra-rénal.*

Un tel mécanisme indirect pourrait être représenté par l'augmentation des acides aminés sanguins, et, en particulier du glutamate (199), au cours des affections hépatiques comme le suggère Martensson (94). Cet auteur a constaté, en effet, qu'une charge en citrate + glutamate détermine chez le lapin une élévation plus importante et plus durable de la citratémie que la même charge en citrate sans glutamate, ce qu'il interprète comme la preuve d'une action inhibitrice du glutamate sur le catabolisme citrique. L'action du glutamate apparaît surtout chez le lapin lorsqu'on fait précéder l'administration de citrate + glutamate d'une ligature de l'artère hépatique, qui détermine chez cet animal une nécrose hépatique. Selon Martensson, il y aurait un parallélisme certain entre l'augmentation des acides aminés sanguins et celle du citrate sanguin au cours des atteintes hépatiques expérimentales, parallélisme qui rendrait vraisemblable une relation de cause à effet entre ces deux phénomènes.

Pour préciser cette éventualité, nous avons réalisé des charges en malate + glutamate et comparé les résultats de ces charges mixtes à ceux obtenus lors de l'injection isolée de malate. Malgré les quantités importantes de citrate formées lors de ces expériences à partir du malate injecté, le rapport citrate/ $\alpha$ -cétoglutarate n'est pas significativement différent lors des charges mixtes de celui noté au cours des charges en malate sans glutamate. Il en est de même des rapports  $\alpha$ -cétoglutarate/succinate et succinate/malate.

*Ces expériences ne permettent donc pas de confirmer les résultats de Martensson, le glutamate n'inhibant pas le catabolisme citrique dans nos conditions expérimentales.* Il est possible que cette constatation négative soit due au catabolisme très rapide du glutamate injecté chez le rat normal. Cependant la comparaison des résultats obtenus chez les rats tétrachlorurés lors de charges en malate seul et en glutamate + malate ne révèle également aucune action inhibitrice du glutamate sur le catabolisme du citrate (voir Fig. 8).

Les rapports des principaux acides du cycle tricarboxylique dans l'urine de ces rats tétrachlorurés sont, en effet, sensiblement identiques lors des charges en *dl*-malate et lors de celles en *dl*-malate + glutamate, sauf le rapport  $\alpha$ -cétoglutarate/succinate qui est plus élevé en cas d'adjonction de glutamate, vraisemblablement par suite d'un processus de transamination d'une partie du glutamate avec l'oxaloacétate ou le pyruvate. Une telle augmentation du rapport  $\alpha$ -cétoglutarate/succinate ne se rencontre pas chez les rats normaux, la différence entre rats normaux et rats tétrachlorurés témoignant vraisemblablement d'une augmentation des transaminations sous l'influence de la lésion hépatique. De nombreux travaux récents ont mis en évidence, en effet,

l'augmentation des transaminases sériques au cours des atteintes hépatiques humaines (30, 196, 197) et expérimentales, en particulier par le tétrachlorure de carbone (100). On peut rapprocher cette constatation de l'augmentation beaucoup plus marquée de l' $\alpha$ -cétoglutarate lors des charges en glutamate chez les malades atteints d'affections hépatiques que lors de ces charges chez des sujets normaux (152).

En dehors des charges en glutamate et malate, nous avons effectué chez le rat tétrachloruré une série de charges destinées à localiser un trouble éventuel du cycle tricarboxylique.

Nous avons effectué en particulier *des charges en  $\alpha$ -cétoglutarate*, le métabolisme de cet acide pouvant a priori être perturbé au cours de l'insuffisance rénale par augmentation des transaminations ou par manque de coenzyme A nécessaire à la formation de succinate à partir de l' $\alpha$ -cétoglutarate. *Les résultats obtenus jusqu'ici ne permettent pas de localiser à ce niveau un bloc éventuel du cycle tricarboxylique.* Le rapport  $\alpha$ -cétoglutarate/succinate est en particulier très voisin chez les rats normaux et chez les rats tétrachlorurés; nous devons signaler cependant que nous avons trouvé chez un rat tétrachloruré une augmentation considérable de ce rapport, qui atteignait 15.4 et pourrait témoigner d'un bloc métabolique dans ce cas unique.

Nous avons réalisé également *des charges en pyruvate + malate* de façon à apporter les deux termes nécessaires à la synthèse du citrate (le malate a été injecté au lieu de l'oxaloacétate en raison de l'instabilité de ce dernier). La considération des rapports entre les acides du cycle tricarboxylique dans l'urine à la suite de ces charges et, en particulier, du rapport malate/citrate, ne révèle pas de différences notables avec le rat normal. Il ne semble donc pas y avoir de perturbation spécifique de l'"entrée" du cycle tricarboxylique, c'est à dire de la synthèse du citrate à partir du pyruvate et de l'oxaloacétate.

*Au total, si nous considérons les résultats obtenus jusqu'ici chez le rat tétrachloruré, il apparaît que l'élimination urinaire des acides du cycle tricarboxylique est supérieure à celle des animaux témoins aussi bien à l'état spontané que lors des charges, sans qu'il apparaisse possible de localiser en un point précis de ce cycle un bloc électif. L'hypothèse la plus vraisemblable pour expliquer cette constatation est l'existence d'un ralentissement des processus métaboliques, ralentissement qui conduirait à une augmentation de tous les acides du cycle aussi bien dans le sang que dans l'urine. Cette hypothèse expliquerait également l'augmentation de la pyruvicémie, dont la fréquence au cours de l'insuffisance hépatique est établie depuis longtemps.*

Un tel ralentissement pourrait d'ailleurs ne pas porter uniquement sur les réactions du cycle tricarboxylique, mais pourrait également être par exemple à la base de la diminution d'utilisation du *D*-lactate mise en évidence par Soffer, Dantes et Sokotka (170) chez les malades atteints d'affections hépatiques.

Nous nous garderons cependant de généraliser et d'admettre qu'il existe

dans toutes les formes d'insuffisance hépatique un ralentissement général métabolique sans bloc localisé au niveau d'une des réactions du cycle tricarboxylique.

Les études enzymatiques ont montré, en effet, la grande variabilité des altérations décelées au cours des insuffisances hépatiques expérimentales selon l'agent responsable de l'atteinte hépatique, le degré des lésions et l'espèce animale considérée.

Le tétrachlorure de carbone détermine ainsi chez la souris, en cas d'administration d'une dose unique, une baisse d'activité de la succinoxydase hépatique (189), baisse qui n'est pas apparente en cas d'administration répétée déterminant une cirrhose hépatique (173). L'activité de la succinoxydase hépatique des rats est, au contraire, normale ou même partiellement accrue lors de l'intoxication tétrachlorurée, ce qui démontre que les modifications enzymatiques dues à un même toxique peuvent être opposées selon l'espèce animale considérée (171). L'éthionine détermine chez le rat une diminution spécifique de l'activité du système oxydant le pyruvate sans que les activités oxydasiques correspondant aux substrats du cycle tricarboxylique soient modifiées (42).

Lors des atteintes hépatiques diététiques, les modifications enzymatiques sont également très variables. Alors que l'activité succinoxydasique hépatique est très réduite dans certains types d'hépatites diététiques (8, 132), cette même activité est normale au cours d'autres hépatites diététiques, qui s'accompagnent d'une perturbation de l'oxydation du pyruvate (121).

Schwarz *et al.* (27, 140, 150) ont récemment individualisé une lésion particulière des tranches de foie de rats recevant un régime déficient en vitamine E avec de l'American torula yeast comme seule source protéique. Cette lésion est caractérisée par la chute progressive de la consommation d'oxygène après 30 à 60 minutes d'incubation et ne s'accompagne pas de perturbations spécifiques de l'un des enzymes du cycle tricarboxylique. Cette lésion, qui pourrait se traduire *in vivo* par des altérations analogues à celles que nous avons constatées chez les rats tétrachlorurés, n'apparaît cependant pas dans les tranches de foie de rats intoxiqués par le tétrachlorure de carbone (28).

La variabilité des résultats de ces études enzymatiques doit inciter à une grande prudence avant de généraliser les conclusions mises en évidence lors d'un type d'atteinte hépatique expérimentale et avant, en particulier, d'admettre que le même mécanisme est à la base des perturbations décelées dans les cas d'hépatite chez l'homme.

#### LES ACIDES DU CYCLE TRICARBOXYLIQUE DANS L'INSUFFISANCE RENALE

Malgré le rôle de premier plan du rein dans le catabolisme des acides du cycle tricarboxylique, on dispose de très peu de données concernant les modifications de ces acides dans les liquides biologiques au cours des insuffisances rénales.



Dans le but de trouver des arguments en faveur de ce rôle métabolique du rein, un certain nombre de dosages de ces acides ont été effectués *dans le sang des animaux néphrectomisés*.

L'acide citrique sanguin s'élève ainsi pour atteindre rapidement des valeurs 4 à 5 fois supérieures à la normale chez le rat et le lapin néphrectomisés et maintenus sous anesthésie, l'élévation étant plus discrète chez le chat (94). Chez l'animal ne subissant qu'une brève anesthésie, l'acide citrique sanguin chute tout d'abord, vraisemblablement sous l'influence du choc opératoire, (52bis), puis s'élève progressivement. La concentration de succinate sanguin ne se modifie guère, par contre, lors de la néphrectomie sans apport de succinate chez le lapin (43); cette concentration s'élève cependant de façon beaucoup plus notable que chez les animaux non néphrectomisés au cours des charges en succinate.

De telles modifications sanguines n'ont pas été retrouvées *au cours des affections rénales humaines*.

Alors que Thunberg (183) avait décelé une augmentation du citrate sanguin dans quelques cas de néphrites, la plupart des autres auteurs s'accordent pour admettre que le citrate sanguin est habituellement normal ou légèrement abaissé au cours des néphropathies humaines (47, 77, 148). Krusius et Vesa (77) trouvent ainsi en moyenne 2.05 mg. % dans 6 cas d'insuffisance rénale (valeurs extrêmes: 1.32-3.16 mg. %) au lieu de 2.18 mg. % chez les sujets normaux. Selon Gey (47), le citrate sanguin est normal dans les cas d'insuffisance rénale sans lésion hépatique associée; cependant, une discrète atteinte hépatique donnerait lieu à une augmentation du citrate sanguin qui ne se serait vraisemblablement pas fait jour en l'absence de l'insuffisance rénale. Nous mêmes avons noté des concentrations de citrate plasmatique variant de 1.33 à 2.02 mg. % dans 15 cas d'insuffisance rénale aiguë.

L'acide  $\alpha$ -cétoglutarique sanguin n'a été dosé qu'au cours de quelques cas de néphropathies. Sa concentration apparaît normale ou abaissée dans l'insuffisance rénale chronique et ceci même dans les formes sévères (12, 47). Alors que le taux d' $\alpha$ -cétoglutarate sanguin avait été trouvé élevé dans un cas d'"urémie," les résultats des dosages que nous avons effectués dans 12 cas d'insuffisance rénale aiguë sont normaux ou légèrement augmentés.

Les modifications urinaires décelées jusqu'ici au cours des affections rénales consistent essentiellement en une diminution de l'élimination du citrate (77, 125, 148). Dans les 6 cas étudiés par Krusius et Vesa (77), l'élimination quotidienne de citrate oscillait entre 80 et 248 mg. au lieu de 200 à 1000 mg. chez les sujets normaux. Chez ces mêmes malades, l'élimination d' $\alpha$ -cétoglutarate était normale, de même que dans le cas étudié par Krebs (70). L'élimination urinaire moyenne de citrate était de 112 mg. par jour (chiffres extrêmes: 25.1 et 292.4 mg.), celle d' $\alpha$ -cétoglutarate de 21.6 mg. (chiffres extrêmes: 11.7 et 28.0 mg.) dans les 12 cas que nous avons étudiés.

Il apparaît donc que *la seule modification des acides du cycle tricarboxylique*



*des liquides biologiques qui soit nettement établie au cours des insuffisances rénales humaines est la diminution de l'élimination urinaire de citrate.*

Cette diminution est-elle due à un simple défaut de perméabilité rénale ou à une perturbation métabolique ?

Bien qu'on possède peu de données sur la clearance normale du citrate (60) et sur la perméabilité de la cellule rénale pour ce composé, on peut supposer que cette perméabilité doit être relativement modérée pour cet acide tricarboxylique, alors qu'elle serait meilleure pour un acide dicarboxylique, comme l'acide  $\alpha$ -cétooglutarique. On pourrait ainsi attribuer à une atteinte de la perméabilité rénale la coexistence d'une élimination urinaire diminuée en ce qui concerne le citrate, normale en ce qui concerne l' $\alpha$ -cétooglutarate.

Pour tenter de savoir si l'insuffisance rénale détermine un simple trouble de la perméabilité pour certains acides du cycle tricarboxylique ou, au contraire, une perturbation de leur métabolisme intra-rénal, nous avons effectué des épreuves de charge en succinate et en malate chez des malades atteints de néphropathies aiguës. Ces épreuves ont été réalisées grâce à l'obligeance du Professeur Hamburger chez des malades de son Service. Il s'agissait de malades atteints de néphropathies post-abortum ou post-transfusionnelles; les charges ont été effectuées chez des malades précédemment anuriques ou fortement oliguriques, d'une part au moment de la reprise de la diurèse, d'autre part à intervalles réguliers au cours de la convalescence ultérieure.

Les charges en succinate faites au moment de la reprise de la diurèse, alors que l'insuffisance rénale est encore sévère, comme en témoigne en particulier le taux élevé de l'urée sanguine, révèlent une perturbation caractéristique. Elles ne déterminent, en effet, aucune augmentation de l'élimination urinaire des acides du cycle tricarboxylique, à l'exception du succinate, dont l'élimination est fortement accrue, et de façon inconstante, de l' $\alpha$ -cétooglutarate, dont la concentration urinaire est alors légèrement augmentée.

Cette perturbation de la réponse à la charge en succinate ne semble pas pouvoir être due uniquement à un trouble de la perméabilité rénale. Le fait que le succinate augmente fortement dans l'urine à la suite de la perfusion montre l'absence d'imperméabilité du rein pour cet acide; il paraîtrait peu vraisemblable qu'il en fût autrement pour les autres acides dicarboxyliques, en particulier pour les acides fumarique et malique. L'absence d'augmentation de leur élimination semble donc témoigner d'un trouble du catabolisme du succinate au niveau du rein. L'augmentation de l' $\alpha$ -cétooglutarate urinaire notée dans certains cas pourrait être en rapport avec l'équilibre stoechiométrique succinate  $\rightleftharpoons$   $\alpha$ -cétooglutarate.

Les charges en succinate réalisées à un stade plus avancé de la convalescence montrent une normalisation progressive de la réponse urinaire, normalisation qui est souvent atteinte alors que le taux de l'urée sanguine est encore élevé. Dans certains de ces cas, on constate, au cours de cette évolution de la réponse à la charge en succinate, un stade transitoire pendant lequel l'élimination

urinaire de citrate augmente sous l'influence de la perfusion de succinate, mais cette augmentation est plus tardive que chez les sujets normaux, le pic d'élimination du citrate se situant dans l'urine émise 12 heures après la charge. La Figure 9 indique, à titre d'exemple, les réponses urinaires lors de trois charges successives en succinate faites chez un même malade en cours d'amélioration progressive d'une insuffisance rénale aigüe.

On constate, également une *perturbation importante de la réponse aux charges en malate* chez ces mêmes malades. Elles déterminent, en effet, une *augmentation notable de l'élimination urinaire de l'acide  $\alpha$ -cétoglutarique*, tandis que la concentration des acides citrique, succinique et fumarique dans l'urine reste inchangée. Les dosages sanguins réalisés parallèlement révèlent également une forte augmentation de l' $\alpha$ -cétoglutarate.

Ayant montré plus haut que le métabolisme du malate injecté à l'homme semble se faire initialement par la voie des transaminations, dont le siège est, selon toute vraisemblance, extrarénal, la constatation chez ces malades d'une élévation de l' $\alpha$ -cétoglutarate sanguin et urinaire lors des charges en malate témoigne de la *persistance de ces processus de transamination*. Elle indique de plus le maintien d'une perméabilité rénale satisfaisante vis-à-vis de l' $\alpha$ -cétoglutarate. L'absence d'augmentation des acides du cycle résultant habituellement du catabolisme de l' $\alpha$ -cétoglutarate peut être interprétée comme le *témoin d'une perturbation métabolique intra-rénale* analogue à celle décelée lors des charges en succinate.

Nous cherchons à l'heure actuelle à localiser cette perturbation métabolique au niveau de l'une des réactions de cycle tricarboxylique grâce, en particulier, à l'étude des rapports entre les différents acides urinaires et à la comparaison des modifications urinaires et des variations du taux sanguin de ces acides au cours des charges.

#### LES ACIDES DU CYCLE TRICARBOXYLIQUE DES LIQUIDES BIOLOGIQUES AU COURS DES AFFECTIONS AUTRES QUE L'INSUFFISANCE HEPATIQUE ET L'INSUFFISANCE RENALE

Diverses modifications des taux sanguin ou urinaire des acides citrique et  $\alpha$ -cétoglutarique ont été signalées au cours d'affections très, diverses, telles que insuffisance cardiaque, (43, 47, 70, 120), hypertension artérielle (153), épilepsie (47, 125), affections gastro-intestinales (194), diverses infections (148, 184), thromboses veineuses (148), maladie de Biermer (125) et de Hodgkin (141), avitaminose B<sub>1</sub> (72, 76, 128 p. 210, 129, 160, 166, 169).

Malgré l'intérêt de l'étude du cycle tricarboxylique au cours du *diabète*, on trouve très peu de données sur les variations des acides de ce cycle dans le sang et l'urine des diabétiques. L'acide citrique sanguin est selon les uns (77, 183) modérément élevé, selon d'autres (47) très variable et sans aucune corrélation avec les modifications de la glycémie et des corps cétoniques. Il ne semble de même exister aucune modification constante du taux de l'acide  $\alpha$ -cétoglutarique sanguin (153). L'élimination de citrate est abaissée en cas

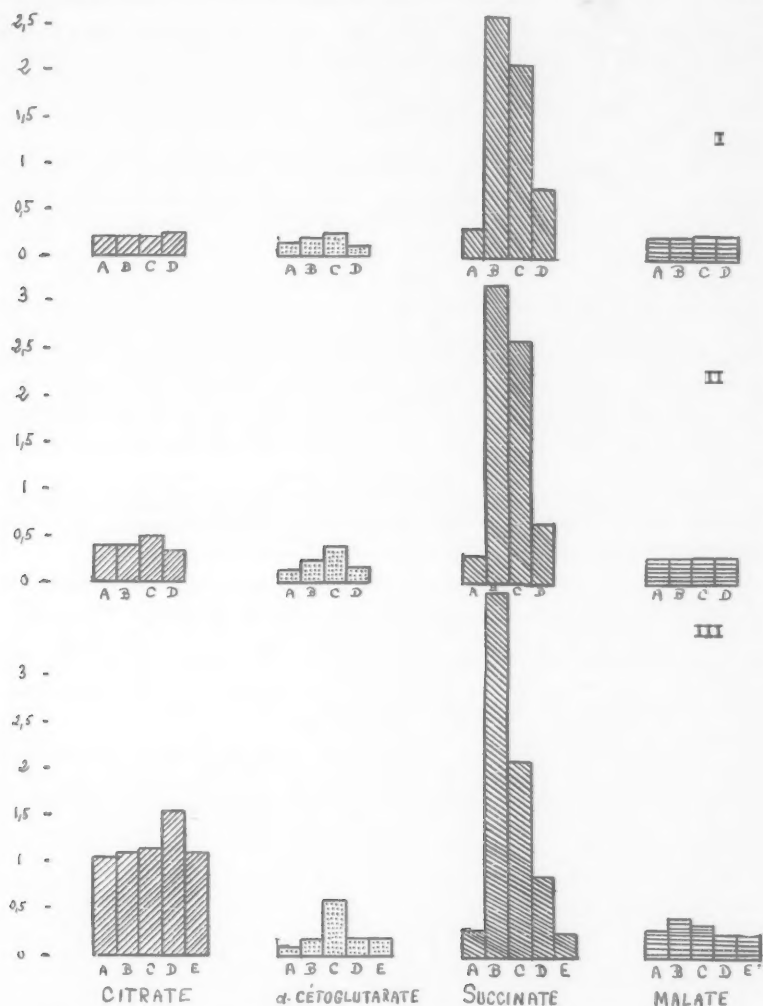


Fig. 9. Modifications Urinaires des Principaux Acides du Cycle Tricarboxylique Déterminées par des Perfusions de Succinate (1 mMole/kg. de poids) chez une Malade Atteinte d'Insuffisance Rénale Aigüe en Voie de Guérison Progressive. Technique de perfusion identique à celle de la Fig. 5. Tous les résultats sont exprimés en mmoles. d'acide pour 1 mg. de créatinine urinaire. I, Au moment de la reprise de la diurèse (urée sanguine: 3.70 Gm./l.). II, 4 jours plus tard (urée sanguine: 3.20 Gm./l.). III, 4 jours après B (urée sanguine: 1.70 Gm./l.). A, avant la perfusion. B, aussitôt après la perfusion. C, 90 minutes après la fin de la perfusion. D, 12 heures après la fin de la perfusion. E, 24 heures après la fin de la perfusion.

de diabète acidotique (13, 77, 125), mais cette baisse pourrait être due à l'acidose elle-même et non à une perturbation spécifique du diabète. L'acide  $\alpha$ -cétoglutarique urinaire, étudié dans quelques rares cas (70, 77) serait élevé, une relation entre cette augmentation et le degré de l'acidose paraissant possible (77).

Le taux du citrate sanguin est généralement normal chez les malades porteurs de différents types de *cancers* (141). Il est intéressant de noter qu'après injection intraveineuse de citrate de sodium, le taux de la citratémie s'abaisse plus rapidement chez les malades atteints de néoplasmes que chez les sujets normaux (64), fait qui pourrait peut-être être interprété comme la traduction des besoins énergétiques accrus de ces malades (47). Le taux de l'acide  $\alpha$ -cétoglutarique sanguin est presque toujours abaissé en cas de cancer (47). Ces documents, pour rares qu'ils soient, montrent l'intérêt de l'étude des modifications des acides du cycle tricarboxylique au cours des états cancéreux, étude que nous entreprenons à l'heure actuelle chez des animaux atteints de néoplasmes expérimentaux ainsi que chez des malades cancéreux et leucémiques.

Nous ne ferons que rappeler ici les faits essentiels concernant les modifications du citrate sanguin et urinaire au cours des affections osseuses, et, d'une façon plus générale, au cours des altérations du métabolisme calcique.

Le tissu osseux est particulièrement riche en citrate (31, 57, 96, 186). Ce citrate osseux ne semble pas représenter une réserve mobilisable sous l'influence de facteurs accroissant son élimination urinaire, tels que l'administration prolongée de bicarbonate, de malate ou de citrate de sodium (29, 86, 155). Il semble, par contre, subir des fluctuations en rapport avec le métabolisme calcique, et notamment avec l'apport de vitamine D.

C'est ainsi que l'avitaminose D diminue la teneur de l'os en citrate (105) ainsi que le taux de citrate sanguin et urinaire (55, 172). L'administration de doses physiologiques de vitamine D à des rats rachitiques élève rapidement le taux du citrate sérique, avant même de déterminer des modifications de la calcémie (23), fait qui suggère que la vitamine D agit directement sur le métabolisme du citrate (57). L'action thérapeutique du citrate dans le rachitisme peut paraître paradoxale, étant donné que l'injection de citrate détermine chez l'animal normal une hypercalciurie et une déminéralisation osseuse (48, 155). En fait, l'action du citrate est opposée lorsqu'il est administré per os et non en injections parentérales : il détermine alors une baisse de la calcémie et de la calciurie, ainsi que la guérison du rachitisme. Selon Yendt et Howard (200) le citrate ingéré complexerait le calcium à l'intérieur du tube digestif, d'où augmentation de l'absorption intestinale des phosphates ; l'accroissement du phosphate disponible permettrait alors la déposition de phosphate de calcium et la reminéralisation des os rachitiques.

La citratémie est abaissée dans l'hypoparathyroïdie (1), élevée au contraire

dans les affections accompagnées d'une hypercalcémie, telles que hyperparathyroïdie et hypervitaminose D (57) ainsi que dans la maladie de Paget (67).

Nous considérerons enfin les modifications de la citraturie au cours de la lithiase calcique urinaire, modifications dont l'intérêt apparaît lorsque l'on considère le rôle physiologique du citrate urinaire sur la solubilisation des sels de calcium.

Plusieurs auteurs ont noté la diminution de la citraturie chez les malades atteints de lithiase urinaire (66, 151, 159). Il faut cependant signaler qu'une telle diminution du citrate urinaire est loin d'être constante chez les malades atteints de lithiase (13). D'autre part, en cas d'infection urinaire, une baisse de la citraturie pourrait être secondaire à un catabolisme bactérien du citrate urinaire (57). Malgré ces restrictions, les rapports entre lithiase calcique et citraturie ont pris un regain d'actualité depuis que Harrison et Harrison (56) ont réussi à reproduire une lithiase calcique expérimentale chez le rat en réduisant l'élimination urinaire de citrate par administration d'un inhibiteur de l'anhydrase carbonique (Diamox). En associant un régime riche en calcium, ces auteurs ont constaté l'apparition d'importantes précipitations calciques intrarénales, tandis que les rats témoins, qui avaient une citraturie importante, ne présentèrent pas de telles calcifications.

#### RENSEIGNEMENTS FOURNIS PAR L'UTILISATION D'INHIBITEURS

Nous ne nous étendrons pas sur les nombreuses études biochimiques basées sur l'utilisation d'inhibiteurs enzymatiques. Etant donné la grande toxicité de ces inhibiteurs, ils ne peuvent être utilisés chez l'homme, si bien que la plupart de ces travaux n'ont pas d'application en chimie clinique. Nous retiendrons cependant quelques faits qui paraissent importants pour la compréhension du métabolisme des acides du cycle tricarboxylique in vivo.

L'action inhibitrice du malonate vis-à-vis de la succinodéshydrogénase, bien établie in vitro, a été largement confirmée in vivo chez l'animal. L'injection de malonate augmente fortement, en effet, l'élimination urinaire du citrate, de l' $\alpha$ -cétoglutarate et surtout du succinate (71, 82, 122). La concentration en succinate s'élève fortement à la suite de l'administration de malonate dans le rein et le foie, tandis qu'elle ne s'élève guère dans le muscle, le cerveau et le sang; les concentrations de malonate les plus fortes sont trouvées dans le sang et le rein, témoignant de leur rôle respectif dans le transport et l'élimination de l'inhibiteur (Busch et Potter, 21).

Le point qui nous paraît devoir être souligné est que l'urine humaine normale contient du malonate, en dehors de toute administration de cet inhibiteur (182). La signification de la présence normale de cet inhibiteur dans l'urine est inconnue.

Depuis les travaux de Buffa et Peters (16, 17), on sait que le fluoroacétate détermine in vivo une importante accumulation de citrate dans nombre de

tissus; cette action du fluoroacétate est due à sa transformation en acide fluorotricarboxylique, lequel a une action inhibitrice sur l'aconitase.

Un point important a pu être établi lors de l'administration de fluoroacétate à l'animal. Cette administration ne détermine, en effet, pas d'accumulation de citrate dans le foie du rat adulte mâle (133), alors qu'au contraire elle élève fortement le citrate intrahépatique chez la femelle (87, 135). Lorsqu'on soumet cependant les femelles au jeûne, l'accumulation de citrate intrahépatique déterminée par le fluoroacétate est aussi faible que chez le mâle, alors qu'à l'opposé l'administration d'un régime déficient en protéines aux rats mâles est suivie d'une forte accumulation de citrate intrahépatique lors de l'injection de fluoroacétate (135). On constate encore une telle accumulation importante chez le rat mâle lorsque l'on fait précéder l'injection de fluoroacétate d'une irradiation par les rayons X (32), ou de l'administration de moutardes azotées (34) ou encore d'une surrénalectomie (33).

Tous ces résultats démontrent le rôle prépondérant de facteurs alimentaires et surtout hormonaux dans le déroulement du cycle tricarboxylique *in vivo* (135).

Signalons pour terminer que nous avons réalisé des charges comportant l'administration de transaconitate + malate + pyruvate. Nous n'avons pas observé d'inhibition du cycle tricarboxylique, contrairement à ce que l'on aurait pu penser d'après les expériences *in vitro* (143, 144, 192). Le transaconitate injecté est éliminé en quasi totalité dans l'urine.

### CONCLUSION

Le cycle tricarboxylique, auquel de nombreuses études *in vitro* ont été consacrées, n'avait encore été envisagé chez l'animal entier et chez l'homme que d'une manière fragmentaire. Grâce à de nouvelles techniques, et particulièrement à la chromatographie sur papier, ces études sont maintenant grandement facilitées.

Cette technique a permis de mettre en évidence l'existence dans l'urine humaine normale d'une vingtaine d'acides organiques, dont tous les acides stables du cycle tricarboxylique, ce qui n'avait pu être fait jusqu'ici. Grâce à des épreuves de charge en divers substrats, l'existence du cycle tricarboxylique a pu être démontrée chez l'homme. Une différence métabolique importante a été constatée entre le rat et l'homme: si le rat semble réagir à l'injection de malate suivant un mécanisme qui peut se superposer au cycle tricarboxylique, l'homme au contraire présente une réaction qui semble impliquer l'intrication du cycle tricarboxylique et de phénomènes de transamination qui ont pour conséquence la précession de la formation d' $\alpha$ -cétoglutarate sur celle de citrate.

Au cours de l'insuffisance hépatique expérimentale par le tétrachlorure de carbone, il existe un accroissement de l'élimination urinaire des acides du cycle tricarboxylique, sans qu'il soit possible de localiser par des épreuves de

charge un bloc en un point précis du cycle. Enfin, au cours de l'insuffisance rénale aiguë chez l'homme, des épreuves de charge en malate et succinate montrent des altérations de la réponse aux charges, altérations qui ne sont pas conditionnées exclusivement par des modifications de la perméabilité rénale.

Ces quelques exemples, pris à dessein dans les trois ordres de recherches possibles: détermination des composés du cycle tricarboxylique dans les liquides biologiques, métabolisme normal chez l'animal entier et chez l'homme, métabolisme pathologique chez l'animal d'expérience et chez l'homme malade, permettent de bien augurer de l'avenir de ce type de recherche.

### SUMMARY

Paper chromatography has greatly facilitated studies of the tricarboxylic cycle in animals and in man. This technic has now shown the existence of approximately twenty organic acids in normal human urine, all stable acids of the cycle. By means of overloading man with various substrates the existence of the tricarboxylic cycle has been demonstrated in humans.

An important difference in metabolism has been noted between man and rat. The rat reacts to an injection of malate by following a mechanism that can be superimposed on the tricarboxylic cycle. Man, on the other hand, seems to follow a reaction which involves the intricacies of the tricarboxylic cycle and the phenomena of transamination. The latter, therefore, shows the formation of  $\alpha$ -ketoglutarate before that of citrate.

In animal experiments with carbon tetrachloride-caused hepatic insufficiency it is shown that there is an increased urinary excretion of acids of the tricarboxylic cycle. In these cases it was not possible to demonstrate any particular point of involvement by means of overloading at any point in the cycle.

During acute renal insufficiency in man it was possible to demonstrate that overloading with malate and succinate resulted in alterations of the cycle. The possibility that the results were caused by any modification of renal permeability has been excluded.

This type of investigation holds great possibilities for future work. The approach is threefold:

1. Determination of the cycle components in biologic fluids.
2. Normal metabolism in animals and man.
3. Abnormal metabolism in both experimental animals and the sick human.

### REFERENCES

1. Alwall, N., *Acta med. scand.* **116**, 337, (1944).
2. Amberg, S., et McClure, W. B., *Am. J. Physiol.* **44**, 453 (1917).
3. Amberg, S., et Maver, M. E., *J. Biol. Chem.* **46**, XV (Proc.) (1921).
4. Ames, R., Sylem, I., et Rapoport, S., *Pediatrics* **6**, 361 (1950).
5. Antonini, E., Casorati, V., et Crifo, S., *Ric. Sci.* **25**, 3035 (1955).
6. Armstrong, M. D., Shaw, K., et Wall, P. E., *J. Biol. Chem.* **218**, 293 (1956).
7. Balassa, G., *Hoppe Seyler's Z. physiol. Chem.* **249**, 217 (1937).



8. Benditt, E. P., Steffee, C. H., Hill, T., et Johnston, T. L., *Fed. Proc.* **8**, 350 (1949).
9. Benni, B., *Biochem. Z.* **221**, 270 (1930).
10. Benni, B., Scherstén, B., et Østberg, O., *Biochem. Z.* **223**, 443 (1930).
11. Bernhard, K., et Andreae, M., *Hoppe Seyler's Z. physiol. Chem.* **249**, 203 (1937).
12. Biserte, G., et Dassonville, B., *Clin. Chim. Acta* **1**, 49 (1956).
13. Boothby, W. M., et Adams, M., *Am. J. Physiol.* **107**, 471 (1934).
14. Bryant, F., et Overell, B. T., *Biochim. Biophys. Acta* **10**, 471 (1953).
15. Buch, H., *Akad. Abhandl.*, Kopenhagen, 1942.
16. Buffa, P., et Peters, R. A., *J. Physiol.* **110**, 488 (1950).
17. Buffa, P., Peters, R. A., et Wakelin, R. W., *Biochem. J.* **48**, 467 (1951).
18. Bunker, J. P., Stetson, J. B., Coe, R. C., Grillo, H. C., et Murphy, A. J., *J.A.M.A.* **157**, 1361 (1955).
19. Busch, H., Hurlbert, R. B., et Potter, van R., *J. Biol. Chem.* **196**, 717 (1952).
20. Busch, H., et Potter, Van R., *J. Biol. Chem.* **198**, 71 (1952).
21. Busch, H., et Potter, Van R., *Cancer Res.* **12**, 660 (1952).
22. Carfagno, S. C., De Horatius, R. F., Thompson, C. M., et Schwarz, H. P., *New Engl. J. Med.* **249**, 303 (1953).
23. Carlsson, A., et Hollunger, G., *Acta physiol. scand.* **31**, 317 (1954).
24. Cavallini, D., Frontali, N., et Toschi, G., *Nature* **164**, 792 (1949).
25. Chalmers, T. C., Iber, F. L., Rosen, H., et Levenson, S. M., *J. Clin. Invest.* **34**, 926 (1955).
26. Cheftel, R. I., Munier, R., et Macheboeuf, M., *Bull. Soc. Chim. Biol.* **33**, 840 (1951).
27. Chernick, S. S., Rodnan, G. P., et Schwarz, K., *Fed. Proc.* **13**, 191 (1954).
28. Chernick, S. S., Moe, J. G., Rodnan, G. P., et Schwarz, K., *J. Biol. Chem.* **217**, 829 (1955).
29. Class, R. N., et Smith, A. H., *J. Biol. Chem.* **151**, 363 (1943).
30. De Ritis, F., Coltorti, M., et Giusti, G., *Boll. Soc. ital. biol. sper.* **31**, 394 (1955).
31. Dickens, F., *Biochem. J.* **35**, 1011 (1941).
32. Du Bois, K. P., Cochran, K. W., et Doull, J., *Proc. Soc. Exp. Biol. Med.* **76**, 422 (1951).
33. Du Bois, K. P., Cochran, K. W., et Zervic, M. M., *Proc. Soc. Exp. Biol. Med.* **78**, 452 (1951).
34. Du Bois, K. P., Derooin, J., et Cochran, K. W., *Proc. Soc. Exp. Biol. Med.* **81**, 230 (1952).
35. Duncan, R. E. B., et Porteous, J. W., *Analyst* **78**, 641 (1953).
36. El Hawary, M. F. S., et Thompson, R. H. S., *Biochem. J.* **53**, 340 (1953).
37. El Hawary, M. F. S., *Biochem. J.* **61**, 348 (1955).
38. Emmrich, R., *Hoppe Seyler's Z. physiol. Chem.* **261**, 61 (1939).
39. Emmrich, R., *Klin. Wochenschr.* **26**, 659 (1948).
40. Ettinger, R. H., Goldbaum, L. R., et Smith, L. H., *J. Biol. Chem.* **199**, 531 (1952).
41. Fasold, H., *Zeitschr. f. Biol.* **90**, 192 (1930).
42. Fischer, G. L., *Proc. Soc. Exp. Biol. Med.* **90**, 153 (1955).
43. Forssman, S., *Acta physiol. scand.* **2**, Suppl. 5, 121 pp., (1941).
44. Friedemann, T. E., et Haugen, G. E., *J. Biol. Chem.* **147**, 415 (1943).
- 44 bis. Frohman, C. E., Orten, J. M., et Smith, A. H., *J. Biol. Chem.* **193**, 277 (1951).
45. Gaffney, G. W., Schreier, K., Di Ferrante, N., et Altman, K. I., *J. Biol. Chem.* **206**, 695 (1954).
46. Gey, F., *Thèse Doct. Sci. Méd.*, Bâle, 1952.
47. Gey, F. K., *Hoppe Seyler's Z. physiol. Chem.* **294**, 128 (1953).
48. Gomori, G., et Gulyas, E., *Proc. Soc. Exp. Biol. Med.* **56**, 226 (1944).
49. Gonoe, J. E., et Templeton, H. L., *Am. J. Dis. Childr.* **39**, 265 (1930).



50. Goodwin, T. W., et Williams, G. R., *Biochem. J.* **51**, 708 (1952).
51. Greenwald, I., *J. Biol. Chem.* **124**, 437 (1938).
52. Grönvall, H., *Biochem. Z.* **220**, 82 (1930).
- 52 bis. Grönvall, H., *Acta ophtalm.*, Suppl. **14**, (1937).
53. Hagelstam, L., *Acta Chirurg. scand.* **90**, 37 (1944).
54. Hallman, N., *Acta physiol. scand.* **2**, Suppl. 4, 136 (1940).
55. Harrison, H. E., et Harrison, H. C., *Yale J. Biol. Med.* **24**, 273 (1952).
56. Harrison, H. E., Harrison, H. C., *J. Clin. Invest.* **34**, 1662, (1955).
57. Harrison, H. E., *Am. J. Med.* **20**, 1 (1956).
58. Hastings, A. B., Murray, C. D., et Sendroy, J., *J. Biol. Chem.* **71**, 723 (1927).
- 58 bis. Hastings, A. B., Mc Lean, F. C., Eichelberger, L., Hall, J. L., et Da Costa, E., *J. Biol. Chem.* **107**, 351 (1934).
59. Henkel, T., *Mölkerei Ztg.* **2**, 259 (1888).
60. Herrin, R. C., et Lardinois, C. C., *Fed. Proc.* **6**, 129 (1947).
61. Huggins, C. B., et Hastings, A. B., *Proc. Soc. Exp. Biol. Med.* **30**, 459 (1933).
62. Hummel, J. P., *J. Biol. Chem.* **180**, 1225 (1949).
63. Isherwood, F. A., et Hanes, C. S., *Biochem. J.* **55**, 824 (1953).
64. Kahle, G., *Klin. Wochenschr.* **27**, 28 (1949).
65. Katz, J., et Chaikoff, I. L., *J. Biol. Chem.* **206**, 887 (1954).
66. Kissin, B., et Locks, M. O., *Proc. Soc. Exp. Biol. Med.* **46**, 216 (1941).
67. Kissin, B., et Kreeger, N., *Am. J. Med. Sci.* **228**, 301 (1954).
68. Koepsell, H. J., et Sharpe, E. S., *Arch. Biochem. Biophys.* **38**, 443 (1952).
69. Kolthoff, I. M., et Laitinen, H. A., *pH and electro-titrations*, New York, Wiley, 1948.
70. Krebs, H. A., *Biochem. J.* **32**, 108 (1938).
71. Krebs, H. A., Salvin, E., et Johnson, W. A., *Biochem. J.* **32**, 113 (1938).
72. Krebs, H. A., *Chem. and Ind.* **57**, 213 (1938).
73. Krebs, H. A., *Ann. Rev. Biochem.* **19**, 409 (1950).
74. Krebs, H. A., *Brit. Med. Bull.* **9**, 97 (1953).
75. Krebs, H. A., *Angew. Chem.* **66**, 313 (1954).
76. Krusius, F. E., et Simola, P. E., *Suomen Kemistilehti* **11 B**, 24 (1938).
77. Krusius, F. E., et Vesa, A., *Acta Soc. Med. Fenn. Duodecim A* **21**, No. 3 (1939).
78. Krusius, F. E., *Acta physiol. scand.* **2**, Suppl. 3 (1940).
79. Kulonen, E., *Scand. J. Clin. Lab. Invest.* **4**, 189 (1952).
80. Kuyper, A. C., et Mattill, H. A., *J. Biol. Chem.* **103**, 51 (1933).
81. Leake, C. D., *Am. J. Physiol.* **63**, 540 (1923).
82. Lee, J. S., et Lifson, N., *J. Biol. Chem.* **193**, 253 (1951).
83. Lehmann, J., *Sv. Fören. inv. med. Förh. Nord. Med.* **8**, 2374 (1940).
84. Lehmann, J., *Sv. kir. Fören. Förh. Nord. Med.* **12**, 3683 (1941).
85. Lehmann, J., *Nord. Med.* **25**, 572 (1945).
86. Leonards, J. R., et Free, A. H., *J. Biol. Chem.* **155**, 503 (1944).
87. Lindenbaum, A., White, M. R., et Schubert, J., *J. Biol. Chem.* **190**, 585 (1951).
88. Lindquist, N., *K. fysiogr. Sällsk. Lund. Förh.* **5**, 17 (1935).
89. Longo, von B., *Z. physiol. Chem.* **1**, 213 (1877).
90. Mc Ardle, B., *Biochem. J.* **60**, 647 (1955).
91. Mc Clure, W. B., et Sauer, L. W., *Am. J. Physiol.* **62**, 190 (1922).
92. Mc Lean, F. C., et Hastings, A. B., *J. Biol. Chem.* **108**, 285 (1938).
93. Marshall, L. M., Orten, J. M., et Smith, A. H., *J. Biol. Chem.* **179**, 1127 (1949).
94. Martensson, J., *Acta physiol. scand.* **1**, Suppl. 2 (1940).
95. Martensson, J., et Thunberg, T., *Acta med. scand.* **140**, 454, (1951).
96. Martensson, J., *Kgl. Fysiograf. Sällskap. Lund. Förh.* **11**, 129, (1941).
97. Martius, C., *Ber. der Physikalisch-Med. Ges. Würzburg* **65**, 79 (1951).

98. Mellone, O., et Yahn, O., *Arg. cir. clin. e exper.* **12**, 369 (1949).
99. Meyer, C. E., et Smith, A. H., *J. Biol. Chem.* **134**, 739 (1940).
100. Molander, D. W., Wroblewski, F., et La Due, J. S., *J. Lab. Clin. Med.* **46**, 831 (1955).
101. Natelson, S., Lugovoy, J. K., et Pincus, J. B., *J. Biol. Chem.* **170**, 597 (1947).
102. Natelson, S., Pincus, J. B., et Lugovoy, J. K., *J. Biol. Chem.* **175**, 742 (1948).
103. Natelson, S., Pincus, J. B., et Lugovoy, J. K., *J. Clin. Invest.* **27**, 446 (1948).
104. Neefe, J. R., *Liver Injury*, Trans. of the 9th Conf., Macy Foundation, 1950, p. 61.
105. Nicolaysen, R., et Nordbø, R., *Acta physiol. scand.* **5**, 212 (1943).
106. Nitzescu, I. I., et Georgescu, I. D., *C. E. Acad. Sci.* **190**, 1325 (1930).
107. Nordbø, R., et Scherstén, B., *Skand. Arch. Physiol.* **63**, 124 (1931).
108. Nordmann, J., Nordmann, R., et Gauchery, O., *Bull. Soc. Chim. Biol.* **34**, 77 (1952).
109. Nordmann, J., et Nordmann, R., *Les résines d'échange ionique en médecine et en biologie*, 104 pp., in *Actualités biologiques*, vol. 1, Paris, Exp. Sci. Fr., 1954.
110. Nordmann, J., Nordmann, R., Gauchery, O., Marty, A., et du Ruisseau, J. P., *Bull. Soc. Chim. Biol.* **37**, 325 (1955).
111. Nordmann, J., du Ruisseau, J. P., et Nordmann, R., *Chromatographie quantitative sur papier des acides du cycle tricarboxylique*. Communiqué au 3ème Congr. Intern. Biochim., Bruxelles, 1955.
112. Nordmann, J., Nordmann, R., du Ruisseau, J. P., et Gauchery, O., *Rev. franç. Et. Clin. Biol.* **1**, 67 (1956).
113. Nordmann, J., et Nordmann, R., A paraître.
114. Nordmann, R., Gauchery, O., du Ruisseau, J. P., Thomas, Y., et Nordmann, J., *Compt. rend. Acad. Sci.* **238**, 2459 (1954).
115. Nordmann, R., Gauchery, O., du Ruisseau, J. P., Thomas, Y., et Nordmann, J., *Bull. Soc. Chim. Biol.* **36**, 1461 (1954).
116. Nordmann, R., Gauchery, O., du Ruisseau, J. P., Thomas, Y., et Nordmann, J., *Bull. Soc. Chim. Biol.* **36**, 1641 (1954).
117. Nordmann, R., du Ruisseau, J. P., et Nordmann, J., *Révélation différentielle de l'acide aconitique en chromatographie sur papier*. Communiqué au 3ème Congr. Intern. Biochim., Bruxelles, 1955.
118. Odin, M., *Sv. Fören. inv. med. Förh. Nord. Med.* **8**, 2374 (1940).
119. Odin, M., *Nord. Med.* **25**, 561 (1945).
120. Ohta, K., *Biochem. Z.* **44**, 481 (1912).
121. Olson, R. E., et Dinning, J. S., *Ann. N. Y. Acad. Sci.* **57**, 889 (1954).
122. Orten, J. M., et Smith, A. H., *J. Biol. Chem.* **117**, 555 (1937).
123. Orten, J. M., et Smith, A. H., *J. Biol. Chem.* **128**, 101 (1939).
124. Osteux, R., et Laturaze, J., *Compt. rend. Acad. Sci.* **239**, 512 (1954).
125. Østberg, O., *Skand. Arch. Physiol.* **62**, 81 (1931).
126. Østberg, O., *Acta Med. Scand. Suppl.* **50**, 172 (1932).
127. Østberg, O., *Biochem. Z.* **226**, 162 (1934).
128. Peters, J. P., et Van Slyke, D. D., *Quantitative clinical chemistry. Interpretations. Vol. 1; (2nd ed.)* Baltimore, Williams & Williams, 1946.
129. Peters, R., *Brit. Med. Bull.* **9**, 116 (1953).
130. Petrucci, D., et Boggio, G., *Boll. Soc. Ital. Biol. Sper.* **30**, 276 (1954).
131. Pincus, J. B., Natelson, S., et Lugovoy, J. K., *Proc. Soc. Exp. Biol. Med.* **78**, 24 (1951).
132. Potter, Van R., et Klug, H. L., *Arch. Biochem.* **12**, 241 (1947).
133. Potter, Van R., Busch, H., et Bothwell, J., *Proc. Soc. Exp. Biol. Med.* **76**, 38 (1951).
134. Potter, Van R., *Proc. Soc. Exp. Biol. Med.* **76**, 41 (1951).
135. Potter, Van R., *Cancer Res.* **11**, 565 (1951).
136. Power, F. W., *Proc. Soc. Exp. Biol. Med.* **33**, 598 (1936).

137. Pucher, G. W., Sherman, C. C., et Vickery, H. B., *J. Biol. Chem.* **113**, 235 (1936).
138. Reid, R. L., et Lederer, M., *Biochem. J.* **50**, 60 (1951).
139. Reinhold, J. G., *Clin. Chem.* **1**, 351 (1955).
140. Rosecan, M., Rodnan, G. P., Chernick, S. G., et Schwarz, K., *J. Biol. Chem.* **217**, 967 (1955).
141. Rottino, A., Hoffmann, G. T., et Brondolo, B., *Proc. Soc. Exp. Biol. Med.* **80**, 339 (1952).
142. Saffran, M., et Denstedt, O. F., *J. Biol. Chem.* **175**, 849 (1948).
143. Saffran, M., et Prado, J. L., *Fed. Proc.* **7**, 182 (1948).
144. Saffran, M., et Prado, J. L., *J. Biol. Chem.* **180**, 1301 (1949).
145. Salant, W., et Wise, L. E., *J. Biol. Chem.* **28**, 27 (1916).
146. Salant, W., Livingston, A. E., et Connet, H., *J. Pharmacol. Exp. Therap.* **10**, 129 (1917).
147. Scherstén, B., *Skand. Arch. Physiol.* **52**, 90 (1929).
148. Scherstén, B., *Skand. Arch. Physiol.* **63**, 97 (1931).
149. Scherstén, B., *Skand. Arch. Physiol.* **74**, suppl. 9, (1936).
150. Schwarz, K., *Proc. Soc. Exp. Biol. Med.* **77**, 818 (1951).
151. Scott, W. W., Huggins, C., et Selman, B. C., *J. Urol.* **50**, 202 (1943).
152. Seitz, W., Enghardt-Gölkel, A., et Schaffry, I., *Klin. Wochenschr.* **33**, 228 (1955).
153. Seligson, D., et Shapiro, B., *Anal. Chem.* **24**, 754 (1952).
154. Seligson, D., McCormick, G. J., et Sborov, V., *J. Clin. Invest.* **31**, 661 (1952).
155. Sendroy, J., *Ann. Rev. Biochem.* **14**, 407 (1945).
156. Sherman, C. C., Mendel, L. B., et Smith, A. H., *J. Biol. Chem.* **113**, 247 (1936).
157. Sherman, C. C., Mendel, L. B., et Smith, A. H., *J. Biol. Chem.* **113**, 265 (1936).
158. Shorr, E., Bernheim, A. R., et Taussky, H., *Science* **95**, 606 (1942).
159. Shorr, E., Almy, T. P., Sloan, M. H., Taussky, H., et Toscani, V., *Science* **96**, 587 (1942).
160. Simola, P. E., *Acta Chem. Fenn.* **11**, 4 et 20 (1936).
161. Simola, P. E., et Kusonen, T., *Suomen Kemistilehti* **11 B**, 22 (1938).
162. Simola, P. E., et Krusius, F. E., *Hoppe Seyler's Z. Physiol.*
163. Sjöström, P., *Acta Chir. Scand.* **79**, Suppl. 49 (1937).
164. Smith, A. H., et Orten, J. M., *J. Biol. Chem.* **124**, 43 (1938).
165. Smith, A. H., Barnes, D. J., Meyer, C. E., et Kaucher, N., *J. Nutrit.* **20**, 255 (1940).
166. Smith, A. H., et Meyer, C. E., *J. Biol. Chem.* **139**, 227 (1941).
167. Smith, L. H., Ettinger, R. H., et Seligson, D., *J. Clin. Invest.* **32**, 273 (1953).
168. Smith, M. J. H., et Taylor, K. W., *Biochem. J.* **55**, XXX (1953).
169. Sober, H. A., Lipton, M. A., et Elvehjem, C. A., *J. Biol. Chem.* **134**, 605 (1940).
170. Soffer, L. J., Dantes, D. A., et Sobotka, H., *Arch. Int. Med.* **62**, 918 (1938).
171. Sprinz, H., et Waldschmidt-Leitz, E., *Hoppe Seyler's Z. Physiol. Chem.* **293**, 16 (1953).
172. Steenbock, H., et Bellin, S. A., *J. Biol. Chem.* **205**, 985, (1953).
173. Stowell, R. E., Lee, C. S., Tsuboi, K. K., et Villasana, A., *Cancer Research* **11**, 345 (1951).
174. Takeda, Y., et Hara, M., *J. Biol. Chem.* **214**, 657 (1955).
175. Taussky, H. H., et Shorr, E., *J. Biol. Chem.* **169**, 103 (1947).
176. Taussky, H. H., *J. Biol. Chem.* **181**, 195 (1949).
177. Taylor, T. G., *Biochem. J.* **54**, 48 (1953).
178. Teschan, P. E., Seligson, D., et McCormick, G. J., *Am. J. Med.* **14**, 531 (1953).
179. Thierfelder, H., et Sherwin, C. P., *Hoppe Seyler's Z. Physiol. Chem.* **94**, 1 (1915).
180. Thomas, K., et Weitzel, G., *Hoppe Seyler's Z. Physiol.* **282**, 170 (1947).
181. Thomas, K., et Weitzel, G., *Hoppe Seyler's Z. Physiol.* **282**, 180 (1947).

182. Thomas, K., et Kalbe, H., *Hoppe Seyler's Z. Physiol.* **293**, 239 (1953).
183. Thunberg, T., *Kgl. Fysiograf. Sällskap. Lund Förh.* **3**, 1 (1933).
184. Thunberg, T., *Acta Med. Scand. Suppl.* **90**, 122 (1938).
185. Thunberg, T., *Acta Path. et Microb. Scand.*, Suppl. **16**, 535 (1938).
186. Thunberg, T., *Kgl. Fysiograf. Sällskap. Lund Förh.* **11**, 42 (1941).
187. Thunberg, T., *Physiol. Rev.* **33**, 1 (1953).
188. Tripod, J., Monchda, A., Jaques, R., et Wirz, E., *Arch. Intern. Pharmacodyn. Therap.* **104**, 121 (1955).
189. Tsuboi, K. K., et Stowell, R. E., *Cancer Res.* **11**, 221 (1951).
190. Verkade, P. E., Van der Lee, J., et Van Alphen, A. J. S., *Hoppe Seyler's Z. Physiol. Chem.* **250**, 47 (1937).
191. Weil-Malherbe, H., et Bone, A. D., *Biochem. J.* **45**, 377 (1949).
192. Weinhouse, S., Millington, R. H., et Wenner, C. E., *Cancer Res.* **11**, 845 (1951).
193. Weitzel, G., *Hoppe Seyler's Z. Physiol. Chem.* **282**, 174 (1947).
194. Welin, G., *Acta Med. Scand. Suppl.* **268**, 132 (1952).
195. Wexler, I. B., Pincus, J. B., Natelson, S., et Lugovoy, J. K., *J. Clin. Invest.* **28**, 474 (1949).
196. Wroblewski, F., et La Due, J. S., *J. Lab. Clin. Med.* **44**, 958 (1954).
197. Wroblewski, F., et La Due, J. S., *J. Clin. Invest.* **34**, 973 (1955).
198. Wroblewski, F., Friend, C., Nydick, I., Rueggseggar, P., et La Due, J. S., *J. Clin. Invest.* **35**, 746 (1956).
199. Wu, C., Bollman, J. L., et Butt, H. B., *J. Clin. Invest.* **34**, 845 (1955).
200. Yendt, E. R., et Howard, J. E., *Bull. Johns Hopkins Hosp.* **96**, 101 (1955).
201. Zappi, F., *Ric Sci.* **23**, 1432 (1953).
202. Zappi, F., *Ric. Sci.* **24**, 103 (1954).

# Enzymes in Clinical Biochemistry

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THE FIRST RECOGNITION of enzymic action was probably that involved in fermentation; certainly the first use of enzymes would seem to have been concerned with the turning of sugar into alcohol. The remarkable difference between the effect of unfermented grape juice and that of wine was remarked upon by many early writers, but no better description is given anywhere, I think, than that by Lucretius who in 55 B.C. wrote, "When the pervasive power of wine has entered into a man and its glow is dispersed through his veins, his limbs are overcome by heaviness; his legs stagger and stumble; his speech is slurred, his mind besotted; his eyes swim; there is a crescendo of shouts, hiccups, oaths; and all the other symptoms follow in due order."

The medieval alchemists used the term "fermentation" interchangeably with "digestion" and "putrefaction." Any reaction in which chemical energy was displayed in some form or other was described as a fermentation. Basil Valentine wrote that when yeast was added to wort "an internal inflammation is communicated to the liquid, so that it raises in itself, and thus the segregation of the feculent from the clear takes place." Johann Becher, in 1669, first recognized that it was alcohol which was formed during the fermentation of solutions of sugar; he distinguished also between fermentation and putrefaction. In 1697, Georg Stahl claimed that fermentation and putrefaction were analogous processes. Antony Leeuwenhoek, in 1680, demonstrated to the Royal Society in London, by means of his microscope, that yeast consisted of discrete bodies. But it was not until 1803, that Thenard claimed that yeast was the cause of fermentation. Schwann and de la Tour in 1836, and Kützing in 1839, regarded yeast as a plant, and alcohol a product of its growth in sugar solutions. Liebig (1839) maintained that alcoholic fermentation was due to instability, a molecular decomposition, set up among the sugar molecules. By 1857, Pasteur had proved fermentation was a metabolic activity of living organisms (yeasts). But the controversy between Liebig

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and Pasteur was never really settled until the work of Harden and Young (1905). It was they who first recognized the important role of phosphates in living processes. Harden showed that active fermentation would take place when the cell-free press juice of yeast was added to a sugar solution, and that an intermediate step in the production of alcohol was the formation of a sugar phosphate. The addition of sodium phosphate accelerated carbon dioxide and alcohol production, and a sugar (fructose) diphosphate, the Harden ester, was isolated from the fermenting solution. It appeared to be this substance which was actively turned into alcohol, carbon dioxide, and free phosphate. The presence in yeast of an enzyme synthesizing sugar phosphate was thus demonstrated. The presence of an enzyme which would liberate free phosphate from the fructose diphosphate was also shown, and this marked the beginning of our knowledge of the phosphatases.

Closely linked with the discovery that sugar is fermented to alcohol by enzymic action, was the discovery by Kirchhoff in 1814, that starch could be converted to sugar by an extract of wheat. Apayen and Persoz (1833) obtained an enzyme of this type, which they called diastase, from malt extract by alcoholic precipitation, and they recognized that saliva also contained an enzyme having the same action. Robiquet in 1830, and Liebig and Wöhler in 1837, studied the hydrolysis of the plant glucoside amygdalin to glucose, and gave the name "emulsin" to the enzymic reagent responsible for the hydrolysis. The demonstration of a starch-splitting enzyme, a diastase, in pancreatic juice, came later, by Danilewsky in 1862, who succeeded in separating it from trypsin.

Discovery of the digestive ferments came from another direction. Early in the seventeenth century van Helmont had suggested that digestion was a chemical rather than a mechanical process. In 1752, Réaumur described how he had fed a buzzard different kinds of food contained in a small perforated metal tube, and how he had found that the food had been dissolved out of the tube, apparently by a solvent action of the stomach juices, when the bird regurgitated the tube. A little later Spallanzani confirmed the observation that food material became liquefied in the stomachs of birds and also of animals. In 1836, Everle and Schwann described the pepsin of gastric juice, and Corvisart, 20 years later, the trypsin of pancreatic juice. Here also was discovered, by Claude Bernard (1856) the fat-splitting enzyme, lipase.

All these early-discovered enzymes are of importance to the clinical chemist; and likewise many others which have only more recently become known, such as the uropepsin of urine, the esterases, the transaminases, the aldolases, and the isomerases. Most of them are important for the study of disease, for diagnoses and prognosis, and for following the effectiveness of various forms of therapy. But it has also been claimed for some enzymes that they may have a field of useful clinical application. Such are certain proteolytic enzymes which have been used by surgeons to remove dead tissue and debris. Since it is with

the phosphatases that my own work has been most concerned, I will spend most time with them, and will deal more briefly with the other enzymes which have proved to be of importance in clinical biochemistry; but since I consider the claims which have been made for the usefulness of enzymes for therapeutic purposes to be rather beyond the scope of this article, I will only refer the reader to two recent reviews by Tagnon (1955) and Howes (1956).

### ALKALINE PHOSPHATASE

Harden's pupil, Robison (Harden and Robison, 1914; Robison, 1922) found that there was a second sugar phosphate, a glucose monophosphate, in the products of fermentation of sugar by yeast. Its calcium salt was freely soluble in water. In studying the action of yeast on this ester, Robison added some of the clear juice to solutions of the calcium glucose monophosphate. After some hours he observed a heavy chalk-like precipitate at the bottom of the solution. This was precipitated calcium inorganic phosphate, and Robison was not slow to recognize that a phosphoric ester-splitting enzyme must be present in the yeast cells which hydrolyzed his glucose monophosphate to free sugar and inorganic phosphate and which precipitated in the presence of calcium ions as insoluble calcium phosphate. Robison was struck by the thought that there might be a similar enzyme present in bony tissue; and indeed that the formation of bone itself might be brought about by a similar process. From young bones he prepared an extract which produced an exactly similar result. It obviously contained a similar enzyme, a phosphatase, which hydrolyzed the glucose phosphate to inorganic phosphate, and gave a precipitate of calcium phosphate. This was the basis of Robison's well-known theory of bone formation which supposes that the bone enzyme in the hypertrophic cells of the tissue where ossification takes place liberates free phosphate from the organic esters of phosphorus contained in the fluids bathing the bone or cartilage, thus giving a local increase in the amount of inorganic phosphorus in solution. By mass action, any increase in the concentration of phosphate ion would lead to a deposition of calcium phosphate in the presence of inorganic calcium. When the bones of rachitic rats were immersed in a solution of calcium glucose phosphate, a dense deposit of calcium phosphate was formed in the matrix of the hypertrophic cartilage, where ossification normally occurs. Since it was only by the action of the bone enzyme that inorganic calcium phosphate could be formed from the glucose phosphate, both the seat of action of the enzyme and its ability to bring about calcification were thus shown (Fig. 1).

Fell and Robison (1929, 1930, 1934) demonstrated the appearance of intense phosphatase activity in cultures of embryonic bone at the time when centers of ossification appeared. Engel and Furuta (1942) showed a similar occurrence of the enzyme in the dental pulp of embryonic teeth. The cartilagenous skeletons of the selachian fishes are almost devoid of phosphatase, whereas their teeth are rich in it (Bodansky *et al.*, 1931; Roche and Bullinger, 1939). A

high phosphatase activity was shown by Huggins (1931) at sites of heterotopic ossification. Botterell and King (1935) found an increase of the enzyme in the sites of fracture repair in the radii of rabbits. Many recent histologic studies have confirmed the distribution of phosphatase in the skeleton and its close association with bone formation (e.g., Bourne, 1943). Robison and Rosenheim (1934) suggested the possibility of a "phosphate cycle" in the bone, whereby phosphoric esters would be synthesized in one stage of a process, and hydrolyzed by the phosphatase in the osteoblasts at a later stage. Roche (1947, 1950) and Gutman and Gutman (1941) gave further expression to this idea, i.e., that a phosphoric ester-synthesizing enzyme (phosphorylase) brings about a phosphorylysis of the glycogen in bone with the inorganic phosphate of the plasma to produce glucose-1-phosphate, which becomes the principal substrate for the bone phosphatase.

The Robison theory of bone formation is no longer accepted by all workers, and other roles for the bone phosphatase have been suggested, e.g., by McKelvie and Mann (1948); Siffert (1951); Neuman and Neuman (1953); and DiStefano, Neuman, and Rouser (1953).

#### PLASMA ALKALINE PHOSPHATASE

Martland and Robison (1926) showed that the blood plasma also contains a phosphatase, similar to that of bone; and Kay (1929, 1930) studied the relative phosphatase activities of plasma from many clinical conditions. A marked elevation was found in generalized bone disease, such as rickets, osteomalacia, Paget's and von Recklinghausen's diseases, and normal or nearly normal levels in other diseases.

#### PROPERTIES OF ALKALINE PHOSPHATASE

The activity of the enzyme is markedly dependent upon pH. It is most active at an alkaline reaction between pH 8 and 10. While the alkaline phosphatase will hydrolyze any primary ester of phosphoric acid, the optimum pH at which it does so depends upon which ester is used as the substrate. Ethylphosphate is hydrolyzed most rapidly at pH 8.1, glycerophosphate at pH 9.4, and phenylphosphate and nitrophenylphosphate at pH 9.8 (King and Delory, 1939). Magnesium ions act as an activator, and likewise certain amino acids (Bodansky, 1946; Roche *et al.*, 1944).

#### METHODS

The original method of Martland and Robinson employed glucose phosphate as a substrate, and a pH of 8.4. Kay (1930) used the more available glycerophosphate as a substrate, but allowed the reaction to proceed at the pH of plasma, i.e., 7.4. At this pH the hydrolysis was so slow that a 48-hour period was required in order to yield an easily measurable amount of inorganic phosphate, determined by the molybdenum blue method. Jenner and Kay (1932) and Bodansky (1933) used glycerophosphate, but at the pH optimum for



hydrolysis; by so doing, and by using very sensitive procedures for the estimation of inorganic phosphate, they were able to cut the hydrolysis time to 3 hours and 1 hour, respectively. Shinowara, Jones and Reinhart (1942), and others, have described similar methods, based on glycerophosphate.

A phenylphosphate method was introduced by King and Armstrong (1934). This was both quicker and more convenient than the glycerophosphate methods. Phenylphosphate is hydrolyzed  $2\frac{1}{2}$  times as fast as glycerophosphate, and a measurable amount of hydrolysis products can therefore be obtained in a lesser time. The very sensitive and accurate method of Folin and Ciocalteu (1927) for determining phenol made it possible to use both less plasma or serum and a shorter incubation period. As modified by King (1945, 1956), the method requires 15 minutes of hydrolysis and 0.2 ml. of plasma. The unit is defined in terms of the number of milligrams of phenol liberated under these conditions. The glycerophosphate methods define their units as equal to the milligrams of phosphorus (inorganic phosphate) liberated.

*p*-Nitrophenylphosphate has been similarly used by Ohmori (1937), King and Delory (1939, 1943), and Bessey, Lowry, and Brock (1946). This substance is almost colorless, but yields the intensely yellow-colored *p*-nitrophenol on hydrolysis, and this can be estimated colorimetrically simply by the addition of sodium hydroxide at the end of the hydrolysis period. Another chromogenic substrate is phenolphthalein phosphate. This is colorless, but hydrolyzes to yield free phenolphthalein, which is intensely red in alkaline solution (Lawford, 1937; King, 1938; Bray and King, 1943; Huggins and Talalay, 1945). Several other substrates have likewise been proposed for estimating plasma phosphatase, e.g., naphtholphosphate, by Seligman *et al.* (1951), adenylic acid by Reynolds, Reynolds, and Walker (1956), and others.

Normal values of plasma alkaline phosphatase are set out in Table 1. Values given by several authors, using different methods, have been included, preference being given to what are considered the most commonly used procedures.

Probably the two most commonly used methods for plasma phosphatase are the Bodansky and the King-Armstrong methods. Representative normal values for adults and children, given in King-Armstrong units, are contained in Table 2, where normal values by several workers, in different countries, are listed.

**Table 1. PLASMA ALKALINE PHOSPHATASES BY SEVERAL METHODS**  
(Units per 100 ml. plasma, normal ranges, according to the different authors' definitions)

Authors	Ranges
Kay (1930)	0.1-0.2
Jenner and Kay (1932)	3-13
King and Armstrong (1934)	3-13
Bodansky (1933)	1.5-5
Shinowara, Jones, and Reinhart (1942)	2.8-8.6
Huggins and Talalay (1945)	3-15

Table 2. NORMAL PLASMA ALKALINE PHOSPHATASE (KING-ARMSTRONG UNITS)

Authors	Ranges
Healthy laboratory workers and others	
American (Reiner, 1953)	4.0 - 10.0
British (King, 1951)	(3.3) 4.5 - 9.5 (12.9)
Canadian (King and Armstrong, 1934)	3.0 - 13.0
Danish (Buch and Buch, 1939)	4.4 - 13.2
Swedish (Arner and Swedin, 1949)	2.0 - 8.0
German civilians (Stern, 1948)	
35 men	4.0 $\pm$ 2.0
33 women	3.0 $\pm$ 2.1
71 children	12.5 (5-28)
Pregnant women, British (Young <i>et al.</i> , 1946)	
First 24 weeks (261 cases)	4.3 $\pm$ 2.0
32 weeks, term (220 cases)	10.5 $\pm$ 3.0
Postpartum (1 week)	10.4 $\pm$ 3.5
Postpartum (6 months)	7.6 $\pm$ 2.5
Infants 0-3 yr., British (Gray and Carter, 1949)	
Normal (56)	17 (11-20)
Clinical rickets (64)	25-40 ( -76)
Early rickets	35

## ALKALINE PHOSPHATASE IN BONE DISEASE

*Rickets.* There is an elevation, often marked, of the plasma phosphatase in clinical rickets (Kay, 1929; Morris and Peden, 1937; Patwardhan, Chitre, and Sukjatankar, 1944; Cantarow and Trumper, 1945; see also the important findings of Gray and Carter, 1949). The findings by these authors are contained in Table 2, and are summarized, together with those for other bone diseases, in Table 3. The findings are given throughout in King-Armstrong units.

*Osteomalacia.* This adult form of rickets also yields enhanced values (see Table 3).

*Osteitis Fibrosa Cystica.* The almost invariably high plasma phosphatase which is found in von Recklinghausen's disease is a reliable diagnostic finding, particularly when taken in conjunction with the high serum calcium (see Table 3).

*Fractures.* The large local increase in the bone phosphatase at the site of a healing fracture (Botterell and King, 1935) is reflected to only a limited extent in the plasma. Values in the tens of units, and sometimes in the twenties, are found (see Peden, 1937).

*Bone Tumors.* Where active replacement, accompanied by osteoblastic activity, is occurring in bone cancer, a raised plasma phosphatase will usually be found. This is the case in osteogenic sarcoma and in metastatic carcinoma. But where there is destruction without replacement, with osteoclastic activity, the phosphatase is not increased, e.g., multiple myeloma and Ewing's tumor (*cf.*, Franssen, Simmons and McLean, 1939; Woodward, 1942; Sullivan, Gutman, 1942; Sunderman, 1942; and Table 3).

Table 3. BIOCHEMICAL FINDINGS IN BONE DISEASE

Bone	Alkaline phosphatase (units/100 ml.)	Phosphorus (mg./100 ml.)	Calcium (mg./100 ml.)
Normal	n (3-13)	n ( $2\frac{1}{2}$ - $4\frac{1}{2}$ )	n (9-11)
Rickets and osteomalacia	h (20-40)	l ( $\frac{1}{2}$ - $2\frac{1}{2}$ )	n
Hypoparathyroidism	n	h ( $4\frac{1}{2}$ -10)	l (5-7)
Hyperparathyroidism	h (20-200)	l ( $\frac{1}{2}$ - $2\frac{1}{2}$ )	h (11-20)
Osteitis deformans	h (20-200)	n	n
Metastatic carcinoma	n-h (10-50)	n	n
Osteogenic sarcoma	h (10-40)	n	n
Multiple myeloma	n	n	n-h (10+)
Fractures	Sl-h (10-20)	n	n

n = normal; l = low; h = high; Sl = slightly; King-Armstrong units.

#### ALKALINE PHOSPHATASE IN JAUNDICE

Roberts (1933) found high concentrations of plasma phosphatase in obstructive jaundice, as well as in generalized bone disease, with only mild increases in nonobstructive jaundice. Armstrong, King, and Harris (1934) produced obstructive jaundice in dogs by ligating or clamping the common bile duct, when very great increases (up to 500 units) in the plasma phosphatase were observed. On relief of the obstruction, the jaundice subsided and phosphatase slowly returned to normal (Fig. 2). Bodansky and Jaffe (1933) had observed similar results, and they have since been confirmed by numerous investigators, e.g., Freeman, Chen, and Ivy, 1938; Gutman, Hogg, and Olson, 1940; Dalgard, 1947, 1949; Jackson, 1952. In contrast to the very high values found in experimental obstructive jaundice, Armstrong and King (1935) found only

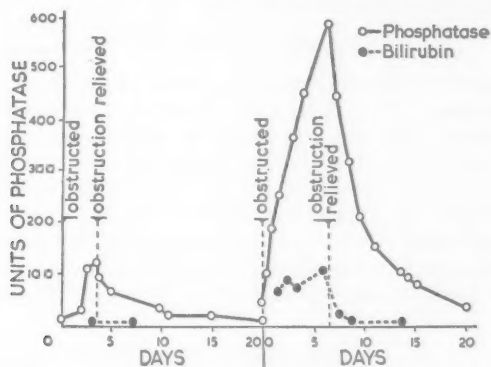


Fig. 2. Obstruction of the common bile duct of the dog. Effect on plasma phosphatase and bilirubin levels (after Armstrong, King, and Harris).

moderate increases in toxic jaundice, and no increase at all in hemolytic jaundice.

In human cases, in obstructive jaundice where the obstruction is nearly complete or long-standing, values of plasma phosphatase of over 30 units and up to 200, may occur, or less than 30 units may be found if the obstruction is partial or intermittent. Values of 15 to 30 units, though occasionally higher, are found in infective hepatitis, cirrhosis, and toxic jaundice; in hemolytic jaundice there is no increase over the normal (Armstrong, King, and Harris, 1934; Herbert, 1935; Maclagan, 1944; Sherlock, 1946). Other authors have reported similar findings, though in different units, e.g., Greene, Shattuck, and Kaplowitz (1934); Meranze, Meranze, and Rothman (1939); Gutman *et al.* (1940); Bodansky and Jaffe (1934); Jaffe and Bodansky (1943); Cantarow and Trumper (1949, 1955).

#### ALKALINE PHOSPHATASE IN SALIVA

Several attempts have been made to correlate the alkaline phosphatase activity of saliva with the occurrence of dental caries, e.g., Glock, Murray, and Pincus, 1938; Lura, 1947; Rae, 1941. But Carter, *et al.*, 1956, failed to find any significant relationship, probably because of the presence of many phosphatase-containing organisms normally present in the mouth. (See the latter authors for list of references).

#### ALKALINE PHOSPHATASE OF THE LEUKOCYTES

Kay (1930) remarked on the presence of an alkaline phosphatase of high activity in the leukocytes. This phosphatase has been studied by several workers, e.g., Wachstein (1946), Cram and Rossiter (1948), Wiltshaw and Moloney (1956). (See the last-named authors for list of references.) No very useful correlation with disease has been discovered.

#### GLUCOSE-6-PHOSPHATASE

This is a special alkaline phosphatase found in the liver (Swanson and Cori, 1948; Swanson, 1950). It has a high degree of specificity for its substrate glucose-6-phosphate, and does not attack the wide variety of primary phosphoric esters against which the plasma alkaline phosphatase is active. Harris and Olmo (1956) have studied the concentration of glucose-6-phosphatase in biopsy and autopsy specimens of liver from normal and diseased children. In glycogen storage disease of the liver the enzyme was almost absent, while it was only slightly subnormal in other liver diseases. In one case of glycogen storage disease of the heart the concentration of the enzyme in the liver was abnormally high.

#### ACID PHOSPHATASE

In 1925, Demuth described a phosphate-splitting enzyme in the urine which was active at an acid reaction, in contrast to the marked alkaline activity of

the bone and plasma phosphatase. Little attention was paid to this work until Kutscher and Wolbergs, in 1935, confirmed the presence of a very high phosphatase activity with an acid optimum in the male urine, with very much less of it in female urine. They traced the source of this enzyme to the prostate gland, where enormous amounts of it were detected, and likewise in the seminal fluid. Since this enzyme differs from that of bone and plasma in being optimally active in the acid range at pH 5, it is referred to as "acid phosphatase" (Fig. 3). It is present in small amounts in blood plasma and in urine; small, that is, in comparison with its enormous concentration in the prostate gland and seminal fluid.

Besides the prostatic acid phosphatase there are other acid phosphatases in various tissues of the body (liver, spleen, kidney, etc.; Roche, 1931; Kutscher and Wolbergs, 1935; Roche, Thoai, and Baudoin, 1942; Behrendt, 1943; Abul-Fadl and King, 1949). King, Wood, and Delory (1945) found a high concentration of an acid phosphatase in red cells, which seemed to contain about 100 times as much as did the plasma. But the red cell acid phosphatase

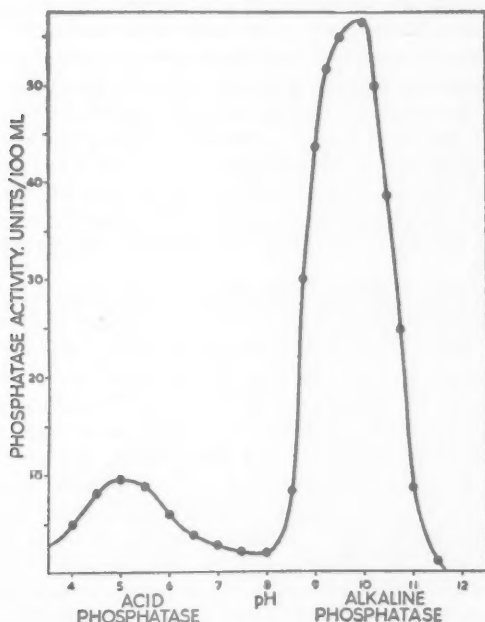


Fig. 3. Variation of plasma phosphatase activity with pH. Plasma from a case of Paget's disease, showing raised alkaline phosphatase and acid phosphatase.

proved to be an entirely different enzyme from the acid phosphatase of plasma, which is derived from the prostate, for the most part, though a small amount of a nonprostatic acid phosphatase, which is distinct from the red cell acid phosphatase, appears to be present in the normal plasma.

Large variations have been observed in the plasma levels of prostatic acid phosphatase, and its determination has been a useful adjunct to the study of prostatic disease. The red cell acid phosphatase has not proved to be a useful subject of investigation, since no marked abnormalities have been found in clinical conditions.

#### METHODS

In principle, the same methods used for alkaline phosphatase can also be applied to the determination of acid phosphatase, provided that an acid buffer of pH 5 is used in place of the alkaline buffer. Fortunately, the alkaline phosphatase is quite inactive at pH 5, and the acid phosphatase is quite inactive at pH 8-10. The phenyl phosphate method of King and Armstrong (1934) is usually used for acid phosphatase determination, with a citrate buffer of pH 5 and 1 hour's incubation. This longer time of incubation, over the 15 minutes used for the alkaline phosphatase, is necessary with normal plasmas or sera, because of the low concentration of acid phosphatase in them; but with plasmas of high acid phosphatase activity, a shorter period of incubation may be employed, provided the results are multiplied by the suitable factor to bring them to the comparable figures of 1 hour's incubation. The acid phosphatase units are equal to the milligrams of phenol hydrolyzed at pH 5 in 1 hour at 37° C. (See Gutman and Gutman, 1940; Huggins, Stevens, and Hodges, 1941; Watkinson *et al.*, 1944; King and Delory, 1948.) The glycerophosphate methods may also be used, and are preferred by some workers (Shinowara *et al.*, 1942; Woodward, 1942).

Herbert (1944, 1946) differentiated prostatic phosphatase from that of normal serum by the ready destructibility of the former with alcohol. Abul-Fadl and King (1948, 1949) found that formaldehyde gives a sharp differentiation between the prostatic and red cell acid phosphatases, the latter being completely destroyed by the inclusion of 0.5% neutral formaldehyde in the reaction mixture, under which conditions the prostatic acid phosphatase is quite unaffected (see also Bensley, Wood, and Lang, 1948). A high acid phosphatase after formaldehyde treatment (above 5 units) strongly suggests a prostatic origin for the enzyme. Abul-Fadl and King likewise found that the prostatic acid phosphatase has a peculiar property of being selectively destroyed by L-tartrate. This property may be made the basis of an estimate of the true prostatic acid phosphatase of the blood plasma by two determinations, one with L-tartrate included, and the other without, the difference in units of phosphatase being taken as the measure of the true prostatic acid phosphatase (*cf.*, Fishman and Lerner, 1953; Bensley, 1956).

## ACID PHOSPHATASE IN THE DIAGNOSIS OF PROSTATIC CARCINOMA

Shortly after Kutscher and Wolbergs' discovery of the prostatic phosphatase, Gutman and his colleagues (1938) began their series of well-known investigations. They found that the enzyme does not appear until puberty, but they were able to produce it precociously in the Rhesus monkey with testosterone propionate. In carcinoma of the prostate, they found the enzyme to be present in the carcinomatous tissue and in the tissue of the skeletal metastases which form as secondaries to the prostatic carcinoma, and which are prominent features of this disease. The small amounts of the enzyme present in the blood plasma were greatly increased in the cases of carcinoma of the prostate with metastases in the bones. In patients without metastases, normal or nearly normal results were obtained.

The normal range of plasma acid phosphatase may be taken as anything up to 5 units of total enzyme, i.e., prostatic and nonprostatic acid phosphatases, and anything up to 3 units of prostatic acid phosphatase by either the formaldehyde or the L-tartrate method. Values above 3 units for prostatic phosphatase should be regarded with suspicion, and anything above 5 units is probably diagnostic of carcinoma of the prostate (or of Paget's disease—see below).

The Gutmans found that a great majority of cases of carcinoma of the prostate with x-ray evidence of metastases had elevated values for the plasma acid phosphatase. The figures obtained by them, and since then by many other workers, may be only slightly elevated above the normal limit of 5 units, or may be greatly enhanced to figures of well over 1000 units per 100 ml.\* An upward trend in the acid phosphatase values indicates an extension of the metastases, and periodic determinations are therefore useful in following the courses of the disease.

White, in 1893, and Cabot, in 1896, found considerable clinical improvement in elderly castrated males suffering from prostatic hypertrophy. Huggins, Stevens, and Hodges (1941) produced great clinical improvement by castrating patients with advanced prostatic cancer. They postulated that prostatic tumors are an overgrowth of adult prostatic epithelial cells, and that prostatic epithelial cells tend to atrophy when androgenic hormones are diminished, e.g., by castration, or when estrogens are administered. Huggins' successful treatment of carcinoma of the prostate by castration and by estrogens has been confirmed by many investigators, e.g., Kahle, Ogden, and Getzoff, 1942; Watkinson *et al.*, 1944; Daniel, Kind, and King, 1954; Hill, 1956. Some representative findings from this laboratory for the prostatic acid phosphatase in the prostatic gland and in blood plasma are given in Tables 4 and 5.

Estrogen treatment both improves the clinical condition of patients with

\*Hudson, Tsuboi, and Mittelman (1955) have also found greatly elevated acid phosphatase values in cases with metastases of the liver from prostatic cancer.

Table 4. ACID PHOSPHATASE IN PROSTATE AND BLOOD

	K.-A. units per 100 Gm. or ml.	
	Range	Average
Prostatic tissue	50,000-250,000	
Seminal fluid (12 normals)	87,000-330,000	206,000
(2 eunuchs)	2,000- 2,400	
Blood plasma		
Males, normal	0-4.6	2.2
Carcinoma of prostate		
(114 cases)	5.3-108	22.4
(Alkaline phosphatase)	(7-32)	(15.7)
Females, normal	0-4	2
Carcinoma of the breast	3.8-6.3	4.9
(Alkaline phosphatase)	(13-22)	(19.0)

Table 5. SERUM ACID PHOSPHATASE ESTIMATIONS ON MALE PATIENTS (IN KING-ARMSTRONG UNITS)

1947-1951	No.
Estimations	808
Patients	527
Raised	
Prostate carcinoma	
4-10 units	7
10-30 units	8
30-40 units	5
40-100 units	4
After prostate palpation	
4-8 units	19
Other diseases (thyroid, bronchus, primary and second carcinomas)	
4-7 units	3
Normal (4 units/100 ml.)	481

From Daniel, 1952.

carcinoma of the prostate with secondaries in the bone, and lowers the level of acid phosphatase in the blood plasma. The determination of the enzyme is therefore a valuable aid in studying the effect of treatment. A rise in acid phosphatase in treated cases may precede clinical evidence of a lessening of the effectiveness of the therapy, and may give the signal for a change of therapy, e.g., in the estrogen or other hormone used (stilbestrol to dienestrol). Determinations of plasma alkaline phosphatase may also be of use, as has been pointed out by Arner and Swedin (1948), and by Wray (1956) (Fig. 4).

Hock and Tessier (1949), Daniel and Van Zyl (1952), Fishman, Bonner, and Homburger (1954), Stewart, Sweetser, and Delory (1950), and Whitmore and Woodward (1955) have found that trauma or even the mild manipu-



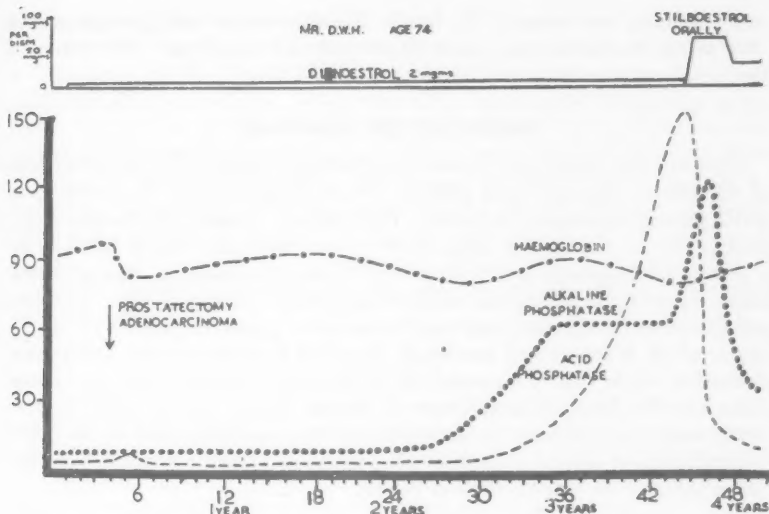


Fig. 4. Acid and alkaline phosphatase levels in the plasma during the course of treatment of a case of carcinoma of the prostate by (a) prostatectomy, (b) dienestrol, and (c) stilbestrol (from Wray, 1956).

lation which occurs in palpation of the prostate in routine clinical examination, may cause a considerable elevation of the plasma acid phosphatase. This appears to be due to expression of some of the enzyme from the prostate gland into the blood stream. Prostatic glands contain extremely large amounts of acid phosphatase and the fluid contained in the cystic cavities of a gland may have amounts of the enzymes of the order of 600,000 u./100 ml. (Fig. 5, p. 511). It would only take a little of such an extremely rich source of acid phosphatase to raise the level in the blood plasma significantly. If an elevated phosphatase is found after routine clinical examination, in which the gland has been palpated during the examination, another determination should be made a few days later, when the enzyme will have been found to return to normal, unless there is malignancy with metastases. Moderately raised values (e.g., 5 to 15 units) may also be encountered in Paget's disease.

#### URINARY EXCRETION OF ACID PHOSPHATASE

Healthy young adults excrete considerable acid phosphatase in their urine (Daniel, Kind, and King, 1954). Part of this appears to come directly from prostatic secretion, and part from renal excretion. The urinary acid phosphatase of patients with carcinoma of the prostate, and with benign prostatic hypertrophy, is much less than that of normal men. Healthy women, and women

suffering from carcinoma of the breast, likewise excrete acid phosphatase in their urine, the values being about 20 per cent of those of men. The source of the female prostate-like acid phosphatase is unknown.

### PHOSPHOHEXOSE ISOMERASE

Phosphohexose isomerase is one of the glycolytic enzymes. In the metabolism of the glucose from glycogen there is first a phosphorylation of glycogen, in which glucose-1-phosphate is formed. There is then a transfer of the phosphate group from the 1 to the 6 position in the glucose molecule. This is followed by a change of the glucose-6-phosphate into fructose-6-phosphate. The phosphohexose isomerase is the enzyme which brings about this last change. Warburg and Christian (1943) suggested that the excessive glycolytic activity of cancer tissue, which Warburg had previously described in several of his well-known researches, might lead to a passage of the glycolytic enzymes from the cancer tissue into the blood plasma. Oscar Bodansky (1954*a* and *b*, 1955) has recently conducted extensive researches to test this possibility, and to see if the determination of plasma phosphohexose isomerase might be a useful biochemical adjunct to the investigation of cancer.

#### METHOD

The determination of phosphohexose isomerase activity is based upon measuring the extent of the conversion of glucose-6-phosphate to fructose-6-phosphate at pH 7.4 and 37° C. in 30 minutes. With the buffered solution of glucose-6-phosphate, 0.5 ml. of serum is incubated and the fructose-6-phosphate formed is estimated by the Selivanoff reaction with resorcinol and hydrochloric acid, a reaction which is specific for fructose. The results are expressed as units which are defined as "the reciprocal of that concentration of serum, expressed as ml. per ml. of reaction mixture, that would cause the formation of 25  $\mu$ g. of fructose as fructose-6-phosphate in 30 min. per ml. of reaction mixture from 0.002M glucose-6-phosphate at pH 7.4 and 37° C."

By this method the mean normal value is 21 units with a standard deviation of 7.0, and an upper limit of 40 units. No difference was observed between the level of the enzyme in normal healthy individuals and persons in a large group of outpatients with miscellaneous noncancerous disease.

#### PHOSPHOHEXOSE ISOMERASE IN CANCER

*Carcinoma of the Breast.* In patients suffering from metastatic carcinoma of the breast there is an impressive degree of correlation between the growth of secondary tumors in the bone and the level of phosphohexose isomerase in the serum. In these cases the excretion of calcium in the urine was also increased, probably as a result of the invasion of the skeleton by the tumor growth. With cases of tumor of the liver the phosphohexose isomerase was also considerably elevated, but without an increase in the urinary calcium. An

illustration of the results obtained with a case of metastatic mammary carcinoma is given in Fig. 6, which is taken from Bodansky's paper (1954b).

*Carcinoma of the Prostate.* Serum phosphohexose isomerase determinations furnish a useful index of the growth or regression of metastases in the bone secondary to carcinoma of the prostate. They correlate well with the acid and alkaline phosphate determinations in most cases. Estimations of the three enzymes furnish a fuller pattern, and a more useful one, of biochemical investigation than is got by any one of them alone. Results from one of Bodansky's cases are reproduced in Fig. 7 from his 1955 paper, where it will be seen that the serum phosphohexose isomerase was always well above normal, and at one time actually above 1000 units in a case of rapidly progressing metastatic prostatic carcinoma.

### ALDOLASE

The next step in glycolysis after the formation of fructose-6-phosphate, mentioned above under phosphohexose isomerase, is the addition of an extra molecule of phosphate to yield fructose-1,6-diphosphate. This fructose diphosphate is then broken in two by the action of the enzyme aldolase into a molecule of glyceraldehyde phosphate and one of dihydroxyacetone phosphate. If phosphohexose isomerase leaks out of the tumor cells into the blood plasma, it might be expected that aldolase, likewise a glycolytic enzyme, would do so too. Sibley and Lehninger (1949a) found an increase of serum aldolase in rats into which they had transplanted tumors; and they also observed that the aldolase was elevated in the serum of some human patients with cancer. Baker and Govan (1953) found high levels of aldolase in the serum of patients with advanced prostatic carcinoma, and that therapeutic measures which reduced the ac-

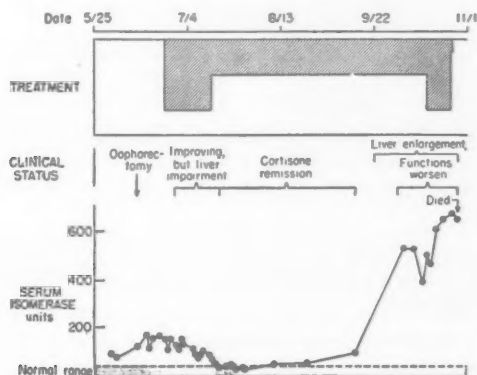


Fig. 6. Serum phosphohexoseisomerase levels in patient with metastatic mammary carcinoma of skeleton and liver (from Bodansky, 1954).

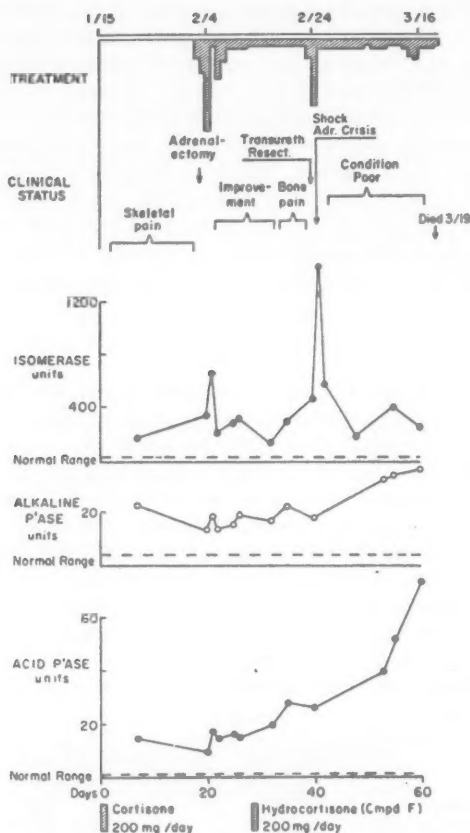


Fig. 7. Markedly elevated serum phosphohexoseisomerase levels in orchiectomized patient with clinical evidence of rapid progression of metastatic prostatic carcinoma. Comparison with serum acid and alkaline phosphatase activities (from Bodansky, 1955).

tivity of the cancer growth also lowered the level of aldolase in the blood serum, as has already been described to be the case with acid phosphatase and phosphohexose isomerase.

#### METHODS

The substrate for aldolase estimations is fructose-1,6-diphosphate, which is now commercially available. In the colorimetric method of Sibley and Lehninger (1949b) a buffered solution of the fructose diphosphate (at pH 7.2) is

incubated with a measured small amount of blood serum at 38° C. for 1 hour. The triose hydrolysis products are determined colorimetrically by adding 2,4-dinitrophenylhydrazine an alkali. In order to prevent isomerization of glyceraldehyde phosphate to dihydroxyacetone phosphate by the triose phosphate isomerase, which is contained in the blood serum, hydrazine is included in the reaction mixture. Baker and Govan (1953) used crystalline rabbit aldolase as a standard of reference. This enzyme is fairly easily prepared by the method of Taylor, Green, and Cori (1948). As is common with work on glycolysis, the results are expressed in terms of the metabolic quotient, i.e., the fructose diphosphate hydrolyzed is considered as if it were a gas, and expressed in terms of microliters ( $\mu$ l.) per milliliter per hour. The range of normal values for human serum aldolase, by this method, is 2 to 9.6, with an average of 5.8 units, each unit being considered as equal to 1  $\mu$ l.

A French modification of this method has been described by Schapira, Dreyfus, and Schapira (1956).

#### ALDOLASE IN CARCINOMA OF THE PROSTATE

Baker and Govan found that 12 of 16 patients with advanced prostatic cancer had increased concentrations of aldolase in the blood serum, with values ranging between 12 and 24 units. Estrogen therapy caused a decrease of the aldolase into the normal range, if the therapy was effective. But if the hormonal therapy failed to produce remission, the level of the aldolase in the serum was usually maintained. The results were in good agreement on the whole, with those for serum acid phosphatase. In a further series of experiments, Baker *et al.* (1953) further explored the effect of diethylstilbestrol on the level of serum aldolase with essentially similar results.

#### ALDOLASE IN MUSCULAR DYSTROPHY

In progressive muscular dystrophy the serum aldolase is likewise elevated (Schapira *et al.*, 1953). Skeletal muscles are a rich source of aldolase, and when muscle cells disintegrate or lose their selective permeability, their substance leaks into the blood stream. It seems that the high serum aldolase activity is simply a consequence of muscle breakdown.

#### ALDOLASE IN MYOCARDIAL INFARCTION

Sibley, Higgins, and Fleisher (1955) found an increase in the serum aldolase in several clinical conditions, including pulmonary infarction, and suggested that a release of the enzyme from damaged cells was the cause. Very recently Volk *et al.* (1956) have reported on the effect of ligating the descending branch of the left coronary artery of dogs. They found a large and rapid postoperative increase of the serum aldolase, and a rough relationship between the myocardial necrosis and the enzyme levels. Eight patients with acute myocardial infarction had considerably raised serum aldolase (Fig. 8), and 5 with

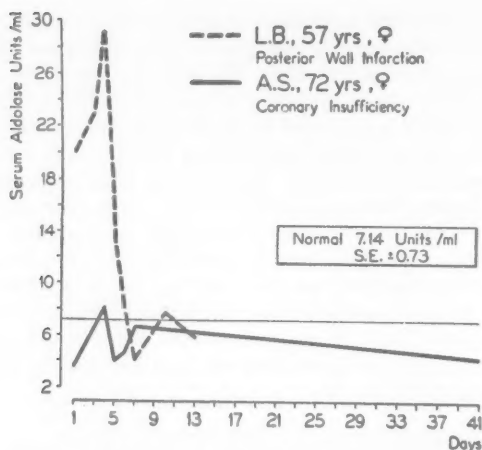


Fig. 8. Daily serum aldolase levels in a patient with acute myocardial infarction and in a patient with coronary insufficiency (Volk *et al.*, 1956).

coronary insufficiency showed normal values (see also the section on transaminase).

#### DIASTASE OR AMYLASE

The names diastase and amylase are used interchangeably for the enzyme which splits the polysaccharide starch to the disaccharide maltose. As was pointed out in the historical introduction, this enzyme is contained in a whole variety of plant and animal fluids and tissues. For the physiologist its principal interest lies in the partial digestion of starch which takes place in the saliva and the complete digestion by the pancreatic amylase acting in the small intestine. For the clinical biochemist its importance lies in the fact that large amounts of it may be found in the urine and in the blood plasma in cases of acute pancreatitis, and lesser subnormal amounts in cases of jaundice. For no very good reason, the enzyme is usually referred to as diastase in the urine, and as amylase in the saliva, pancreatic juice, and blood.

#### URINARY DIASTASE

The estimation of urinary diastase is of some importance in diseases of the pancreas, particularly in acute pancreatitis, in which higher values than normal are found. The units used are those of Wohlgemuth. They are given by the number of milliliters of 0.1 % starch solution digested by 1 ml. of urine in 30 minutes. Expressed in these terms, the normal values are 6–30 units per ml. In pancreatic abnormalities the values may rise to over 100 units.

*Method.* Different dilutions of the urine, buffered to pH 6.1 with phosphate solution, are mixed with a 0.2% solution of starch. After incubation at 37° C., iodine is added to the samples, and the last tube in which no color is produced is taken as giving the dilution at which the starch is just completely digested. (Urinary diastase is often reported in terms of the output of the enzyme for 24 hours.) Some characteristic results are as follows: acute pancreatitis, 200 units/ml.; chronic pancreatitis, 10 to 50 units/ml.; neoplasm invading pancreas, 30 to 100 units/ml.

#### PLASMA AMYLASE

The determination of plasma amylase is of importance in the diagnosis of acute pancreatitis in which high values are obtained. It has the advantage over the estimation of urinary diastase in that it is unnecessary to wait for the collection of a 24-hour specimen of urine. A high plasma amylase is also found in salivary gland inflammation and mumps, and low values in liver disease and pancreatic insufficiency.

The standard method for estimating plasma amylase is the method of Somogyi (1941), and is dependent upon the hydrolysis of starch under standard conditions of pH, temperature, and time, and the estimation of the reducing sugar (maltose) which is formed. The unit is defined in terms of the amount of reducing sugar liberated from the starch by the plasma under these precisely stated conditions. Other rather simplified methods are, however, usually used. In one method the time in minutes which it takes a small amount of plasma completely to digest a buffered starch solution, until no more blue color is given with iodine, is accurately measured with a stopwatch. In another method a small amount of plasma is incubated at 37° C. with 0.4 mg. starch, and the loss in blue color which the starch gives with iodine solution is taken as a measure of the extent to which the starch has been digested by the amylase. The unit is here defined as the amount of amylase which will destroy 5 mg. starch in 15 minutes. But in both methods the conditions are so adjusted that the units are almost identical with those of Somogyi. Normal values for plasma amylase were determined by King and Wootton (1956) to be between 71 and 209 units.

*Pancreatitis.* Determination of plasma amylase is important in the diagnosis of acute pancreatitis, and in distinguishing this condition from other causes of acute abdominal pain. In many acute cases the rise of plasma amylase, though very high, is only short-lived and soon falls toward normal; the blood sample should therefore be taken soon after the onset of the pain. Such a case is illustrated in Fig. 9. Values of more than 1000 units per 100 ml. are usually found in acute pancreatitis. McCorkle and Goldman (1942) studied 43 cases of acute pancreatitis. The lowest plasma amylase they encountered was 225 units, the highest was 2500 units, and the average was 670 units. In more than 200 other cases of acute abdominal disease they found that the serum amylase

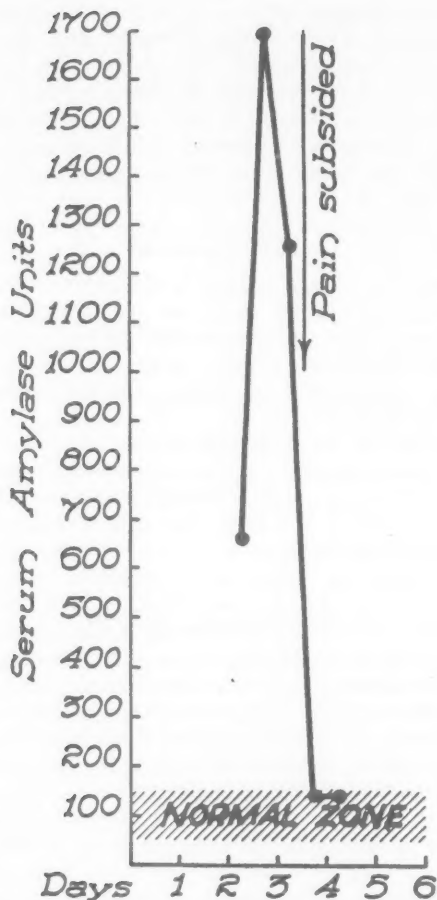


Fig. 9. Sharp rise and fall of the serum amylase levels in a case of transient acute interstitial pancreatitis associated with acute alcoholism (from McCorkle and Goldman, 1941).

was a valuable aid to rule out the possibility of acute pancreatitis. In 42 cases of acute cholecystitis, an additional diagnosis of acute pancreatitis was made as the result of finding a high serum amylase. Some other conditions may have increased serum amylase values, but they are seldom of the magnitude found in acute pancreatitis, e.g., cholecystitis, perforated peptic ulcer, and intestinal obstruction. Burnett and Ness (1955) investigated 12 cases of acute



pancreatitis, all of which had values of over 1000 units. In most of the non-pancreatic conditions less than 4000 units were found, though in 2 cases of perforated ulcer and 2 of intestinal obstruction over 1000 units were determined.

*Mumps.* Ninety per cent of patients investigated by Candel and Wheelock (1946) and by Warren (1955) had high levels of serum amylase. In the absence of any evidence of pancreatitis the elevation appears to be related to the severity with which the parotid glands are affected. Warren (1955) has pointed out that the high level of serum amylase may persist after the parotitis has disappeared, and he thinks that there may be involvement of the intestinal cerozymogenic glands. In support of this, it is of note that Wallman and Vidor (1955) found that 8 cases of mumps meningoencephalitis showed raised levels of amylases in the absence of any evidence of salivary gland or pancreatic gland disease. This is of importance, since the etiology of meningoencephalitis may be difficult, and it may not be easy to distinguish it from other viral infections like nonparalytic poliomyelitis.

### LIPASE

Ingested fats (triglycerides, phospholipids) are largely broken down in the small intestine through the hydrolytic action of the enzyme lipase present in the pancreatic secretion. It was shown by Willstätter that there is also a lipase present in the gastric secretion, but this enzyme is relatively unimportant for the digestion of fat. Lipase causes the hydrolysis of the ester linkages between the fatty acids and the glycerol of the triglycerides (neutral fat) and the phospholipids. Partial hydrolysis results in the formation of diglycerides, monoglycerides, and free fatty acids, and of glycerol and free fatty acids if the hydrolysis is complete. It follows that the determination of lipase activity may be in terms of the estimation of any one of the hydrolytic products. Since the free fatty acids are the most easily determined, it is usually the increase in their concentration in the hydrolyzing mixture which is taken as a measure of the amount of lipase present.

Because lipase is principally formed by the pancreas, it is with pancreatic function that determinations of lipase activity are most useful. Like amylase it may get into the blood in considerably increased concentrations above the small amounts normally present. Its determination has not, however, been as widely employed as that of amylase. This is partly due to the greater difficulty of estimating its concentration in the blood plasma accurately, and partly to the fact that the increases in its concentration in pancreatic disease are not as dramatic as those for amylase.

### METHODS

Substrates which have been used for the estimation of lipase activity are the naturally occurring neutral fats (triglycerides) of which olive oil has been

the most commonly employed; tributyrin, which is easily obtainable in the purified or semipurified state; the ethyl esters of long-chain fatty acids, such as ethyl palmitate; Archibald's water-soluble derivative of sorbitan mono-laurate (Tween 20); and Seligman and Nachlas's (1950)  $\beta$ -naphthyl laurate. A disadvantage of the triglycerides (olive oil or tributyrin) is their insolubility in water. It is necessary to bring them into a state of high emulsification so that the particles may be sufficiently small for the enzyme easily to get at them; this has usually been done by the use of such emulsifying agents as bile acids and vegetable gums. At alkaline reactions, where lipase acts most readily, the rate of hydrolysis is increased by the presence of proteins, soaps, and calcium ions. The first two seem to act as activators, and the calcium to remove the products of hydrolysis from solution by precipitating the insoluble calcium soaps of the fatty acids liberated. Ethyl palmitate and ethyl laurate and  $\beta$ -naphthyl laurate suffer from the disadvantage that they are attacked not only by lipase, but by the nonspecific esterases of the blood, which may be present in considerable amounts in certain clinical conditions, and may give a high rate of hydrolysis which would be falsely attributed to lipase. The same objection holds for the use of tributyrin, for, as Ravin and Seligman (1951) pointed out, the specificity of a substrate for lipase depends upon the chain link of the fatty acids in it. Although these substrates may give elevated serum lipase values in the blood plasma of patients with pancreatitis, they do not uniformly do so, and there appears to be at the present time no satisfactory substitute for olive oil (triolein, the trioleic acid ester of glycerol) as a substrate for lipase in blood plasma. As the amount of lipase in blood plasma is small, and the rate of hydrolysis of olive oil is slow, the amounts of free fatty acid liberated in the given time are not very great, and most methods have therefore used a 24-hour incubation in order to get sufficient fatty acid for an accurate titration. Using the method of Cherry and Crandall (1932), several investigators have found a large proportion of patients with acute pancreatitis to have elevated plasma lipase values (e.g., Johnson and Bockus, 1940; Janowitz, 1952). Bunch and Emerson (1956) have recently compared the 24-hour olive oil lipase methods of Northman, Pratt, and Benotti (1948) with a new 4-hour olive oil method, in which the liberated fatty acids are titrated with a sensitive electrometric method. The olive oil is emulsified in water with gum acacia by means of a homogenizer, the emulsion mixed with a phosphate buffer at pH 7.5, and the mixture incubated with 1 ml. of blood serum for 4 hours at 37° C. The lipolytic hydrolysis is stopped by the addition of an excess of alcohol-ether mixture, and the free fatty acids are titrated with 0.1N sodium hydroxide to a pH of 10.65, using a pH meter. The results are expressed as "units" which are equal to the number of milliliters of 0.05N NaOH required to titrate the free fatty acid hydrolyzed in 4 hours by 1 ml. of serum. There was good agreement between the 24-hour method and the quicker more sensitive 4-hour procedure.

## PANCREATITIS

By the Bunch and Emerson method a large number of healthy control individuals, both male and female, gave serum lipase levels ranging between 0.06 and 0.87 units, with an average of 0.31. Cases of acute pancreatitis showed levels of between about 1.5 and 3 units during the course of an attack with slow subsidence towards normal during recovery. Patients with nonpancreatic disease yielded normal results, except for cholelithiasis, which also had a somewhat elevated serum amylase. While it seems unlikely that lipase estimations will rival those of amylase as a biochemical investigation in pancreatitis, there is no doubt that it is a useful additional determination.

Lipase is claimed to persist at a high level in the serum and to fall much more gradually, following an attack of acute pancreatitis, than does amylase. This is an extra reason for making both determinations, since a diagnosis can often be made because of a high serum lipase, when the amylase is found to be normal or nearly normal. Moreover, the serum lipase is often elevated in cases of chronic pancreatitis, whereas the serum amylase remains essentially normal. Raised values have been reported in about half the cases of carcinoma of the pancreas and of the ampulla of Vater, and in some cases of liver disease (Cantarow and Trumper, 1955).

In multiple sclerosis raised serum lipase values were found by Brickner (1930), Crandall and Cherry (1932), and Lesny and Polacek (1951); although this has been questioned by Brickner (1935), Swan and Myers (1937), and Richards and Wolff (1940).

## TRYPSIN

Trypsin has been referred to in the historical introduction as one of the early digestive enzymes to be described. It is the principal proteolytic enzyme of pancreatic juice, and hydrolyzes the proteins and their semisplit products passed on from the stomach into their constituent amino acids. Unlike amylase and lipase, trypsin does not get into the blood in sufficient amount to make its estimation there a useful investigation. Peptide-splitting enzymes (peptidases) have recently been found in human blood by Fleisher (1953, 1954, 1956). But it cannot be said yet whether they will prove of any interest to the clinical biochemist.

Measurement of the concentration of the proteolytic enzymes, e.g., trypsin, in the duodenal juice, is often of use in the study of pancreatic dysfunction, particularly in children. The incomplete digestion of protein may be suspected from examination of the feces; but it is more satisfactory to determine the actual enzyme content of a sample of the aspirated juice. In pancreatic insufficiency the concentration of the enzyme may be considerably reduced.

## METHODS

It is usual to estimate the content of proteolytic enzyme by studying the rate at which it digests (hydrolyzes) casein. The amount of hydrolysis products

formed from casein in a given time is taken as a measure of the amount of the enzyme present. The hydrolysis may be followed by determining the amount of liberated peptides or amino acids in terms of the increase of titratable carboxyl or amino groups. For the former, the Willstätter method which titrates the liberated carboxyl groups in alcoholic solution with potassium hydroxide is employed, or the Sorensen amino acid method in which the amino groups are destroyed by formaldehyde, making the carboxyl groups available for titration by sodium hydroxide in aqueous solution; for amino groups the Van Slyke method is used.

In another method, Charney and Tomarelli (1947) employed a chromophoric protein substrate, sulfanilamide-azocasein. This substance is precipitated by trichloroacetic acid, but its potentially colored digestion products remain in solution. When the enzyme action is stopped the residual undigested azocasein is precipitated by the trichloroacetic acid, and the amount of color in the filtrate, after making alkaline, can then be taken as the measure of the extent of the hydrolysis, and hence of the enzyme concentration.

In feces, as well as duodenal juice, the trypsin activity is often estimated by observing its ability to liquefy gelatin. The gelatin is incubated with several dilutions of the feces and sodium bicarbonate buffer. Those dilutions which have sufficient proteolytic enzyme present cause liquefaction of the gelatin. The more enzyme there is in the feces, the higher dilution in which the liquefaction takes place. These methods are described by King and Wootton (1956).

#### TRYPSIN OF DUODENAL JUICE

Agren, Lagerlöf, and Berglund (1936) used a secretin test of pancreatic function. They give details of the injection of the hormone, the aspiration of the duodenal juice, and its chemical analysis for trypsin, amylase, bicarbonate content, etc. The mean values and variability of normal function are fully described. In acute pancreatitis they found a diminution of the amylase content, without any diminution of the trypsin and bicarbonate. In pancreatic insufficiency both trypsin and amylase were diminished, and the bicarbonate not much affected. (See also Cantarow and Trumper, 1955.)

Bodian (1952) showed that the duodenal juice of normal children (297 cases) digested gelatin in the manner described above, at dilutions of the juice greater than 1 in 50; whereas cases of fibrocystic disease of the pancreas digested it either not at all, or at dilutions of only 1 in 6 or 1 in 12.

#### PROTEOLYTIC (TRYPSIN) ACTIVITY OF FECES

After the first 3 days of life, the feces of babies and young children have a high proteolytic activity. By the age of 12 years, proteolytic activity of the stool has decreased markedly, whereas that of duodenal juice remains fairly constant. For this reason, it is only in infants that the investigation of fecal proteolytic activity is useful. Payne (1952) showed that the feces of

most normal babies digest gelatin at a dilution of 1 in 100 or greater, whereas babies with fibrocystic disease of the pancreas digest it only with a dilution of less than 1 in 50.

### PEPSIN

Pepsin is the only important enzyme of the gastric juice. It partially hydrolyzes proteins to proteoses, peptones, and peptides, the hydrolysis to the constituent amino acids being completed by trypsin in the duodenum. Pepsin can be estimated in the gastric juice, in relation to gastric function or to anemia, but it is not considered usually to be a very useful determination. Pepsin is almost never absent if free HCl is present in the gastric juice. For this reason, examination of the juice is usually confined to determinations of free and total HCl and of abnormal constituents.

### METHODS

The classic method for estimating gastric pepsin is by the use of the so-called "Mett tube." Egg white is drawn up into narrow-bored glass tubes, sealed at the end with plasticine or sealing wax, and the albumin coagulated by brief cooking in boiling water. The tubes may then be broken up into lengths of about 2 inches, and the ends sealed off with sealing wax to preserve the coagulated albumin from evaporation and contamination from the air. When one of these tubes, from which the seal at the end has been removed, is immersed in gastric juice, the pepsin digests the albumin and, so to speak, eats its way up into the tube. The number of millimeters of albumin digested out of the tube, in a given time, is taken as a measure of the peptic activity of the juice. This was the method which was most used in the classic researches of the Pavlov school.

The digestion of casein may also be used for estimating pepsin, and the amount of digestion may be estimated in terms of the trichloroacetic acid-soluble nitrogen formed in a given time, as determined by the Kjeldahl method, by titration with potassium hydroxide in alcohol by the Willstätter method, or by titration with sodium hydroxide after the addition of formaldehyde by the Sorensen method.

A very convenient colorimetric method (Anson and Mirsky, 1932) employs hemoglobin as a substrate, and estimates the liberated tyrosine groups by the Folin and Ciocalteu phenol method.

### ACHLORHYDRIA AND ACHYLIA

In achlorhydria, as the name implies, there is an absence of hydrochloric acid in the gastric secretion. This is often apparent and not real, when hydrochloric acid, absent from the resting juice or from that brought forth by an ordinary test meal, is elicited by the injection of histamine. Achylia is the term applied to the absence of both free hydrochloric acid and pepsin from the gastric juice. The determination of pepsin is of chief importance in

distinguishing between achlorhydria and achylia. Histamine injections may also be used for distinguishing between apparent and true achylia. In pernicious anemia, the determination of pepsin is an important investigation, since true achylia often appears in this condition. It may also be present in severe secondary anemia, in subacute combined degeneration of the cord, and in children it may follow an attack of gastroenteritis.

### UROPEPSIN

Although pepsin has not been demonstrated in the blood, as far as I am aware, it does seem that some of the enzyme must enter the blood stream, probably directly from the secreting cells of the stomach, since a pepsin-like enzyme is found in the urine. It seems highly probable that this enzyme is in fact the same pepsin as is found in the gastric juice; at any rate it has the same properties, i.e., maximum activity at pH of 1-2, dependence upon the presence of chloride ions for its activity, and a hydrolytic activity against proteins which stops short of the final degradation. This enzyme is usually called uropepsin.

In an attempt to overcome the necessity for intubation to obtain gastric juice for analysis, some investigators have thought to employ the estimation of uropepsin as a substitute. While a few authors have claimed success with the method, the majority of workers have not found it to be a very useful determination in those conditions for which a gastric test meal is useful. Thus, unreliable results have been reported in gastric and duodenal ulcer and in gastric carcinoma. Moreover, an increased excretion of uropepsin in the urine has been noted as a result of stress, and the amount of enzyme excreted therefore appears to be at least partially under adrenocortical control. Nacheles *et al.* (1956) studied the effect of the administration of cortisone on the excretion of uropepsin in aged female subjects. There was some indication that cortisone had an effect, but the results were so variable that no definite conclusions could be drawn. (See the review by Bucher, 1947, on uropepsin.)

### METHOD

The estimations are made on samples of 24-hour collections of urine. The Anson and Mirsky method referred to above is used. One milliliter of urine and 5 milliliters of 2.5% hemoglobin solution, both adjusted to pH 1.5 are incubated at 37° C. for 1 hour. The hydrolysis is stopped with trichloroacetic acid, and the tyrosine groups estimated in the filtrate by the addition of the Folin and Ciocalteu phenol reagent followed by sodium hydroxide. The blue color is compared against an appropriate tyrosine standard, and the results are calculated as micromols of tyrosine liberated. The values are then usually calculated to a 24-hour basis.

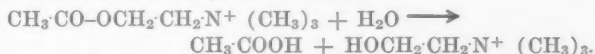
### UROPEPSIN AS A TEST OF GASTRIC SECRETION

It has been stated above that uropepsin determinations do not correlate well with the results of analyses on specimens taken in a gastric test meal.

Levy and Levine (1956) have made multiple determinations on patients before and after partial gastrectomy. They concluded that the estimations did not give a good test of gastric secretory activity. There was no consistent excretion pattern before or after operation, and vagotomy had no effect on uropepsin secretion. After removal of part of the stomach, there is a decrease in the uropepsin in some, but not all, the patients; but where there was a decrease it was not proportional to the amount of stomach tissue removed. They concluded that uropepsin excretion was not proportional to gastric secretion.

### ACETYLCHOLINE ESTERASE

Acetylcholine esterase catalyzes the reaction



Its name is usually abbreviated to choline esterase. The principal role of its substrate, acetylcholine, is as a chemical transmitter of the nerve impulses. It was discovered by the distinguished Austrian physiologist, Loewi. Acetylcholine is liberated at the end plates during the passage of a nerve impulse, and is promptly destroyed by the choline esterase. The partial and purified enzyme has also been obtained from the plasma and erythrocytes of mammals. The enzyme preparations from both plasma and erythrocytes hydrolyze acetylcholine, but that from the plasma also hydrolyzes esters derived from the aliphatic alcohols; whereas that in the erythrocytes will hydrolyze acetylcholine, and is inactive against the other esters. It has therefore become the custom to speak of the plasma choline esterase as "pseudo" choline esterase and that of the erythrocytes as "true" choline esterase (see Mendel and Rodney, 1943).

The action of choline esterase is strongly inhibited by the alkaloids physostigmine (eserine), prostigmine, and atropine, even at such high dilutions as micromolar. It is also inhibited, irreversibly, by the organophosphorus insecticides.

Estimations of the choline esterase have been principally used as tests of liver function, and of poisoning by the organophosphorus insecticides, of megaloblastic anemia, and in a miscellaneous set of other clinical conditions including vitamin B<sub>1</sub> deficiency, thyrotoxicosis, and diabetes.

### METHODS

From the above equation it will be seen that the two products of hydrolysis of acetylcholine by the esterase are choline and acetic acid. While it would be possible to estimate the choline liberated, as a measure of the hydrolysis, it is much easier to estimate the increase in the acidity of the incubating mixture, due to the liberation of the acetic acid. An early method used for the plasma choline esterase was the so-called "continuous titration." To an



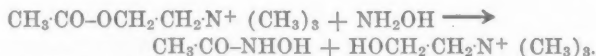
unbuffered aqueous solution of acetylcholine was added a drop of phenol red indicator and a small measured amount of blood plasma. Dilute sodium hydroxide solution was added dropwise until the mixture was just pink to the indicator, i.e., at a *pH* of approximately 7. As the enzyme acted on the acetylcholine, acetic acid was liberated, the *pH* of the mixture fell, and the indicator turned from pink to yellow. Further dropwise additions of sodium hydroxide were made during the course of half an hour, in order to keep the mixture just pink to phenol red. The amount of sodium hydroxide added during this time gave a measure of the concentration of the enzyme present (Stedman, Stedman, and Easson, 1932). A recent modification of this method is given by Brown and Bush (1950), who investigated cases of parathion poisoning.

Choline esterase can likewise be estimated in the Warburg apparatus. A solution of acetylcholine in sodium bicarbonate buffer is incubated with a measured amount of blood. The acetic acid released acts on the sodium bicarbonate to liberate carbon dioxide, and this is measured in the Warburg manometers (Ammon, 1934; Callaway, Davies, and Rutland (1951)).

Michel (1949) described a very simple and convenient electrometric method, which enabled a large number of estimations to be made quickly. The method has been variously modified by several workers, e.g., Aldridge and Davies (1952), Marchand (1952), and MacDonald, Pollard, and Gropp (1952), and has been widely used, particularly by the investigators of insecticide poisoning. Essentially, it consists in allowing the enzyme contained in a measured amount of blood to hydrolyze acetylcholine in a weakly buffered solution; as acetic acid is liberated from the acetylcholine, the *pH* of the mixture drops, and can be easily measured with a *pH* meter. Results are expressed in units which are defined as "*pH* units" equal to the drop in *pH* multiplied by 100. Callaway *et al.* (1951) found the red cell choline esterase levels of healthy individuals to fall in the range of 75 to 142 units.

Colorimetric *pH* indicators have been used instead of a *pH* meter, in similar methods, by Reinhold, Tourigny, and Yonan (1953) and Shibata and Takahashi (1953).

A sensitive colorimetric method for choline esterase in red blood cells or whole blood has been described by Fleisher and Pope (1954). Hestrin (1949) showed that acetylcholine reacts with hydroxylamine to form acethydroxyamic acid:



The acethydroxyamic acid forms a red color with ferric salts in acid solution, and the intensity of the color is proportional to the concentration of acetylcholine. This procedure furnishes a very sensitive method for following the rate of disappearance of acetylcholine as it is hydrolyzed by the esterase.

It is usual to use blood plasma or serum for the estimation of choline esterase



in the investigation of liver disease. This determination, therefore, represents the "pseudo" choline esterase for the most part, although there is some "true" esterase in the plasma. For investigating the effects of insect poisoning on the workers in the factories where it is made and on the users of it in the field, it is important to determine the "true" choline esterase, and it is therefore preferable to use separated erythrocytes, although whole blood is also often used.

#### ACETYLCHOLINE ESTERASE IN JAUNDICE

The serum choline esterase was found by McArdle (1940) to be considerably reduced from the average normal value of 78 units. On sera of normal persons, McArdle found all values to be over 50; children averaged 105 units. In cases of cirrhosis he obtained an average figure of 34 units, in hepatitis 35 units, and in obstructive jaundice 64 units, where it is therefore only very slightly reduced. This test has been investigated by several workers and has been modified, e.g., by the inclusion of fluorophosphonate as a specific inhibitor of the enzyme (*cf.*, Wescoe *et al.*, 1947). Borhaus, Seudamore, and Kark (1950, 1953) have made extensive use of the measurement of serum choline esterase in the study of diseases of the liver and biliary system, but the method has not had as wide an application as other tests of liver function in common use, such as bilirubin, cholesterol, alkaline phosphatase, and the protein flocculation tests.

#### BLOOD AND ERYTHROCYTE CHOLINE ESTERASE IN ORGANOPHOSPHORUS INSECTICIDE POISONING

All the organophosphorus compounds used as insecticides inhibit the enzymes that hydrolyze acetylcholine. Their toxic properties are due to a consequent accumulation of acetylcholine in the body, caused by a failure of the enzyme to destroy it. The poisoning may be mild and without symptoms, or it may be severe and even fatal. Abrams, Hamblin, and Marchand (1950) reported that at least 30 deaths had occurred among workers handling these compounds. So far as I am aware no fatal case has occurred in Great Britain, although a case of poisoning was reported by Milles and Salt (1950). (See also Goldblatt, 1950; Bidstrup, 1950; Marchand, 1952; Barnes and Davies, 1951.) The latter authors state that observations on animals have shown that the amount of choline esterase circulating in the blood may be lowered by as much as 80 per cent without symptoms of poisoning appearing. They examined 139 specimens of blood from 30 factory and 50 agricultural workers, the former being engaged in the packing and preparation of the insecticides and the latter in the spraying of fruit with them. Abnormally small levels of choline esterase were found in only 12 of these investigated cases, and the departures from normal were only very slight, and no serious case of poisoning was observed. Nevertheless the determination is an important clinical test which makes it possible to detect the absorption of organophosphorus insecti-

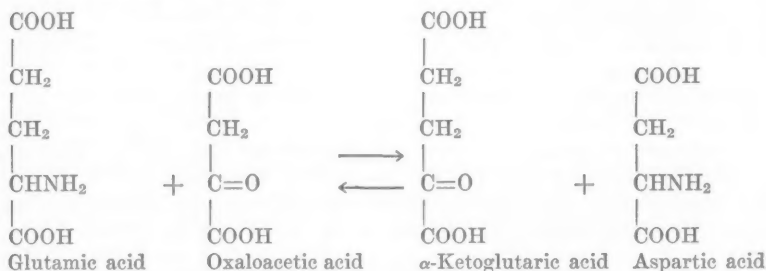
cide poisons before enough has been absorbed to produce a dangerous intoxication.

#### ERYTHROCYTE CHOLINE ESTERASE IN ANEMIA

It was observed by Sabine (1940, 1951) that there is a decrease of choline esterase in both red cells and blood plasma in pernicious anemia. (See also Meyer *et al.*, 1948; Scudamore *et al.*, 1951). Pritchard and Weisman (1956) studied the effect of the increased erythrocyte production of normal pregnancy on the red cell choline esterase content.

#### TRANSAMINASE

The mechanism of the formation of amino acids was formerly thought to involve a reaction between ammonia and an  $\alpha$ -keto acid. But in 1934, Herbst and Engel demonstrated a typical transamination by heating together  $\alpha$ -aminophenylacetic acid and pyruvic acid. The amino group of the  $\alpha$ -aminophenylacetic acid appeared to be exchanged with the keto group of the pyruvic acid, thereby forming alanine and phenyl glyoxylic acid, the latter decomposing into benzaldehyde and  $\text{CO}_2$ . Braunstein and Kritzman (1937) furnished the first evidence that animal tissues contain enzymes that bring about such reactions. And it seems highly likely that a transamination between an  $\alpha$ -amino acid and an  $\alpha$ -keto acid represents a biologic pathway in the formation of amino acids. Cohen (1939, 1940) found two transaminating systems in heart muscle. The one catalyzed the formation of alanine and  $\alpha$ -ketoglutaric acid from glutamic acid and pyruvic acid; and the second  $\alpha$ -ketoglutaric acid and aspartic acid from glutamic acid and oxaloacetic acid. The equation for the second of these is as follows:



The other possible reaction from these substances, i.e., that between aspartic acid and pyruvic acid, could be brought about by a combination of the two reactions, but the presence in liver of a specific transaminase for the direct reaction between aspartic and pyruvic was also reported.

La Due, Wroblewski, and Karmen reported in 1954, that the glutamic oxaloacetic transaminase of blood serum was considerably elevated in myo-

cardial infarction and hepatocellular disease. Karmen, Wroblewski, and La Due (1955) found the enzyme to be present in all specimens of human blood serum examined. Rudolph, Dutton, and Schaefer (1955) produced experimental infarctions by ligation of the coronary, pulmonary, renal, splenic, and mesenteric vessels, and found considerable increases of the transaminase activities of the sera. The amount of transaminase in the serum probably reflects the amount of tissue involved in the pathologic process and its concentration of the enzyme.

#### METHODS

Karmen *et al.* (1955) devised a method for measuring the serum glutamic oxaloacetic transaminase by determining spectrophotometrically the oxidation of reduced diphosphopyridinenucleotide (DPNH) which occurs when malic dehydrogenase is added to  $\alpha$ -ketoglutaric acid and aspartic acid. These are transformed, according to the above equation, into glutamic acid and oxaloacetic acid when their buffered solution is incubated with the transaminase contained in a measured amount of blood serum. The method thus depends upon a reduction by DPNH of oxaloacetic to malic acid. It consists of a simple spectrophotometric measurement and is quick and easy to carry out. The results are expressed in units per milliliter of serum. Using this method, Chinsky, Shmagranoff, and Sherry (1956) found an average value of 20 ( $\pm 8$ ) units per milliliter of serum in 50 determinations on 15 healthy young adults, with a range of 7 to 40 units. Kattus *et al.*, (1956) found a mean serum transaminase level of 20 units, with a range of 16 to 24, in 11 normal persons; and nearly normal levels in hospital patients not suspected of having acute myocardial infarction or liver disease. They regard 40 units as being the upper limits of normal.

Tonhazy, White, and Umbreit (1950) devised a method of estimating transaminase in which the oxaloacetic acid, which is formed along with the glutamic acid, from  $\alpha$ -ketoglutaric acid and aspartic acid under the action of the enzyme, is converted to pyruvic acid with aniline citrate. The pyruvic acid is then determined through its reaction with 2,4-dinitrophenylhydrazine. This method lacked specificity because several  $\alpha$ -ketoacids react with dinitrophenylhydrazine. Henley and Pollard (1956) modified the Tonhazy procedure by converting the oxaloacetic acid to pyruvic acid by heating, and then determining the latter with lactic acid dehydrogenase, through measuring the oxidation of DPNH spectrophotometrically. The reduced diphosphopyridinenucleotide (DPNH) has a much higher optical density at 340  $m\mu$  than has diphosphopyridinenucleotide (DPN). The reduction in optical density can therefore be taken as a measure of the oxidation of DPNH, which is in turn a measure of pyruvate (through its reduction to lactate) and hence of the oxaloacetic acid from which it was formed. With a buffered mixture of  $\alpha$ -ketoglutaric and aspartic acids 1 milliliter of serum is incubated. The en-

zyme action is stopped, and the oxaloacetic acid formed is converted to pyruvic acid by heating in a boiling water bath. To the cooled mixture is added DPNH and lactic acid dehydrogenase. Readings are taken in a spectrophotometer immediately before adding the dehydrogenase and finally when the reaction is complete, as is evidenced by no further decrease in optical density. Results are expressed in terms of the micromols of oxaloacetic acid produced per milliliter of plasma per hour. As determined on 43 specimens from normal subjects, the average normal value with this method is 1.34 ( $\pm 0.12$ )  $\mu\text{mol./ml./hr.}$

#### TRANSAMINASE ACTIVITY IN ACUTE MYOCARDIAL INFARCTION

La Due *et al.* (1954) found that in 30 patients with acute transmural myocardial infarction the transaminase rose to levels 2 to 20 times normal within 24 hours, returning to normal within 3 to 6 days. Normal values were found in patients with heart disease uncomplicated by acute myocardial infarction, acute febrile and chronic infectious diseases, uremia, pulmonary infarction and neoplastic diseases.

Kattus *et al.* (1956) found an elevation of transaminase in the serum of 13 of 14 patients with myocardial infarction. The level of the enzyme in the serum began to rise 6 to 12 hours after the onset of the infarction, the peak was reached in 24 to 36 hours, and a decline to normal had begun by the fifth or sixth day. The peak values reached varied between 100 and nearly 600 units. In a typical case, the transaminase level began at about 35 units, rose to nearly 250 units 24 hours later, and had gradually sunk to less than 40 units by the end of 7 days. Patients with other forms of heart disease had normal values.

Chinsky *et al.* (1956) found 108 high transaminase values in 117 cases of acute myocardial infarction. The peak level often reached values of over 400 or 500 units at about 24 hours after the beginning of pain. This slow return to the normal range lasted for 3 to 6 days. Patients with the anginal syndrome did not have elevated values of serum transaminase. Figure 10 shows the transaminase levels in the sera of 84 cases of myocardial infarction observed after the onset of pain. It is taken from the work of Chinsky *et al.* (1956).

#### TRANSAMINASE IN JAUNDICE

La Due *et al.* (1954), Wroblewski and La Due (1954), Kattus *et al.* (1956), and Chinsky *et al.* (1956) all found high to very high values for serum transaminase in hepatitis. Similarly with cirrhosis, although not as high values were reached. In obstructive jaundice, increases were observed but they were variable and not very great. The usefulness of this investigation in the study of hepatic dysfunction remains to be fully explored.

#### COPPER OXIDASE

Holmberg and Laurell (1948) isolated from the blood of both humans and pigs a blue copper protein with a molecular weight of about 150,000, the copper

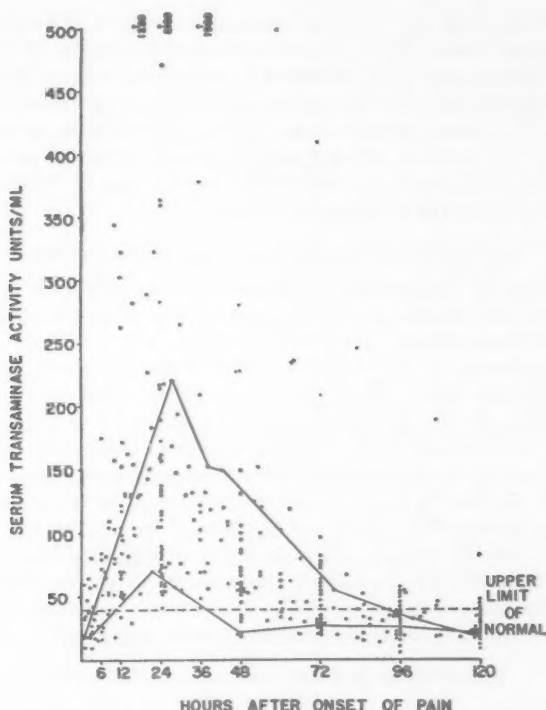


Fig. 10. Serum transaminase levels observed after the onset of pain in 84 instances of proved myocardial infarction (from Chinsky, Shmagranoff, and Sherry, 1956).

being combined with an  $\alpha$ -globulin. The name ceruloplasmin was given to this compound. It was shown by Holmberg and Laurell, in 1951, that it can act as an oxidase, with *p*-phenylenediamine. Scheinberg and Gitlin (1952) showed that there was a lowered copper oxidase level in the blood in Wilson's disease, i.e., in hepatolenticular degeneration. Bearn and Kunkel (1954) and Cumings, Goodwin, and Earle (1955) confirmed these findings.

#### METHOD

Ravin (1956) has developed a simple colorimetric procedure for the estimation of serum copper oxidase using *p*-phenylenediamine as the substrate. Under the action of the enzyme, the *p*-phenylenediamine is oxidized to produce a dark lavender color by the serum of a normal person, whereas the serum from one of hepatolenticular degeneration produces only a very pale lavender or a pale flesh color. The test is convenient and very easy. With 3 ml. of

acetate-buffered (pH 6.4) *p*-phenylenediamine solution, 0.1 ml. of serum is incubated for 1 hour at 37°. The enzyme action is stopped by the addition of 1 ml. of sodium azide; and a control is carried through at the same time as the test with the inclusion of sodium azide. The colors are read at 530  $m\mu$ , and the results recorded as extinctions, i.e., the differences in optical densities between tests and controls. Normal subjects showed values of serum oxidase, in these terms, of 0.1 to 0.3 (average, 0.18) and in cases of Wilson's disease, values ranging from 0.01 to 0.04 were found.

#### COPPER OXIDASE IN HEPATOLENTICULAR DEGENERATION

The biochemical investigations useful in the investigation of Wilson's disease are a demonstration of an increased urinary excretion of amino acids (Uzman and Denny-Brown, 1948) and of copper (Cumings, 1951) and a low serum ceruloplasmin (copper oxidase) level (Scheinberg and Gitlin, 1952; Bearn and Kunkel, 1952). These determinations have been very difficult and time-consuming, by the methods used in the past, and it is possible that the new simple procedure of Ravin for copper oxidase may prove to be a very valuable procedure for investigating this serious condition, particularly in its early stages. A lowered serum copper oxidase has been the most consistent and striking abnormality among the biochemical investigations of Wilson's disease; and, indeed, it may be the most firmly established biochemical defect for having been present the longest. By estimations of the copper oxidase it may be possible to detect cases with incipient hepatolenticular degeneration before the disease has manifested itself in a convincing manner.

#### SERUM COPPER IN ACUTE MYOCARDIAL INFARCTION

Vallee (1952) has shown a persistent elevation of serum copper after acute myocardial infarction, and Adelstein, Coombs, and Vallee (1956) found a direct relation between copper concentration, ceruloplasmin, and *p*-phenylenediamine oxidation. This copper oxidase enzyme, like aldolase and transaminase, may therefore prove to be a useful investigation in myocardial infarction.

#### SUMMARY

Enzymes are used in modern medicine for diagnosis and prognosis, and, to a limited extent, for certain therapeutic purposes. Digestive enzymes in the stomach, small intestine, and pancreas, and in the stool, have a relation to gastric and pancreatic function and to absorption. Uropepsinogen, as well as pepsin, has a relation to anemia and gastric secretion. Serum lipase is raised in acute pancreatitis. Amylase, both in urine and in plasma, is similarly raised and may also be of diagnostic value in mumps. Alkaline phosphatase is a test for the differential diagnosis of jaundice. It is markedly elevated in generalized bone disease. Plasma acetylcholinesterase is decreased in cases of poisoning by modern insecticides like parathion; it is also used

as a test of hepatic function. Serum acid phosphatase, aldolase, and phosphohexoseisomerase are elevated in prostatic and breast cancer, and are a good index of the growth of metastatic bone tumors and also of the effect of treatment. Transaminase is raised in jaundice and in myocardial infarction. Copper oxidase is markedly lowered in hepatolenticular degeneration.

## REFERENCES

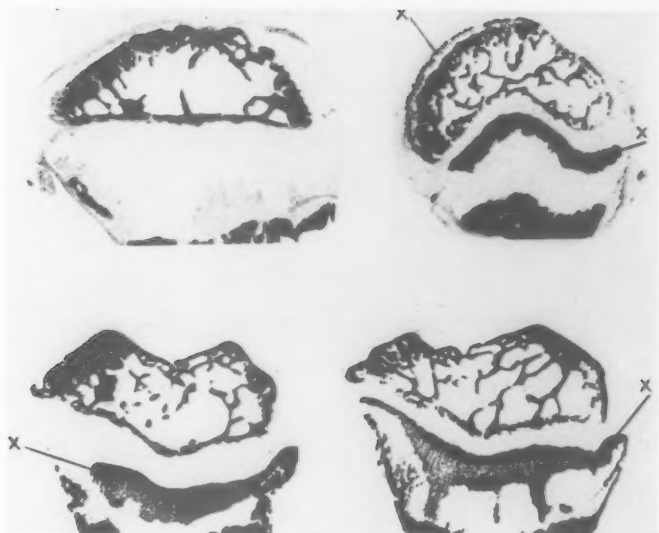
1. Abrams, H. K., Hamblin, D. O., and Marchand, J. F., *J. Amer. Med. Asso.* **144**, 107 (1950).
2. Abul-Fadl, M. A. M., and King, E. J., *J. Clin. Path.* **1**, 80 (1948).
3. Abul-Fadl, M. A. M., and King, E. J., *Biochem. J.* **45**, 51 (1949).
4. Adelstein, S. J., Coombs, T. L., and Vallee, B. L., *New Engl. J. Med.* **255**, 105 (1956).
5. Agren, G., Lagerlöf, H., and Berglund, H., *Acta Med. Scand.* **90**, 224 (1936).
6. Aldridge, W. N., and Davies, D. R., *Brit. Med. J.* **1**, 945 (19 ).
7. Ammon, R., *Pföger's Arch. Ges. Physiol.* **233**, 486 (1934).
8. Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.* **16**, 59 (1932).
9. Armstrong, A. R., and King, E. J., *Canad. Med. Asso. J.* **32**, 379 (1935).
10. Armstrong, A. R., King, E. J., and Harris, R. I., *Canad. Med. Asso. J.* **31**, 14 (1934).
11. Arner, O., and Swedin, B., *Acta Chir. Scand.* **97**, 137 (1949).
12. Baker, R., and Govan, D., *Cancer Research* **12**, 141 (1953).
13. Baker, R., and Govan, D., Huffer, J., and Cason, J., *J. Clin. Endocrin.* **13**, 383 (1953).
14. Barnes, J. M., and Davies, D. R., *Brit. Med. J.* **ii**, 816 (1951).
15. Bearn, A. G., and Kunkel, H. G., *J. Clin. Invest.* **31**, 616 (1952).
16. Behrendt, H., *Proc. Soc. Exp. Biol. N. Y.* **54**, 268 (1956).
17. Bensley, E. H., *Amer. J. Clin. Path.* **26**, 247 (1956).
18. Bensley, E. H., Wood, P., and Lang, D., *Amer. J. Clin. Path.* **9**, 742 (1948).
19. Bernard, C., *Memoire sur le Pancreas, etc.* Paris, Balliere. (1856).
20. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.* **321**, 164 (1946).
21. Bidstrup, P. L., *Brit. Med. J.* **2**, 548 (1950).
22. Bodansky, O., *J. Biol. Chem.* **101**, 93 (1933).
23. Bodansky, O., *J. Biol. Chem.* **165**, 605 (1946).
24. Bodansky, O., *Cancer*, 1191 (1954a).
25. Bodansky, O., *Cancer*, 1200 (1954b).
26. Bodansky, O., *Cancer* **8**, 1087 (1955).
27. Bodansky, O., and Jaffe, H. L., *Arch. Intern. Med.* **54**, 88 (1934).
28. Bodansky, O., Bakwin, R. M., and Bakwin, H., *J. Biol. Chem.* **94**, 551 (1931).
29. Bodian, M., *Fibrocystic Disease of the Pancreas*. London, Heinemann, 1952.
30. Botterell, E. H., and King, E. J., *Lancet* **i**, 1267 (1935).
31. Bourne, G., *Quart. J. Exp. Physiol.* **32**, 1 (1943).
32. Bray, J., and King, E. J., *J. Path. Bact.* **55**, 315 (1943).
33. Brickner, R. M., *Arch. Neurol. Psychiat.*, Chicago, **34**, 466 (1930).
34. Bricker, R. M., *Arch. Neurol. Psychiat.*, Chicago, **34**, 466 (1935).
35. Brown, H. V., and Bush, A. F., *Arch. Industr. Hyg.* **1**, 633 (1950).
36. Buch, I., and Buch, H., *Acta Med. Scand.* **101**, 211 (1939).
37. Bucher, G. R., *Gastroenterology* **8**, 627 (1947).
38. Bunch, L. D., and Emerson, R. L., Unpublished data, (1954).
39. Bunch, L. D., and Emerson, R. L., *Clin. Chem.* **2**, 75 (1956).
40. Burnett, W., and Ness, T. D., *Brit. Med. J.* **2**, 770 (1955).
41. Cabot, A. T., *Ann. Surg.* **24**, 265 (1896).
42. Callaway, S., Davies, D. R., and Rutland, J. P., *Brit. Med. J.* **2**, 812 (1951).

43. Candel, S., and Wheelock, M. C., *Ann. Intern. Med.* **25**, 88 (1946).
44. Cantarow, A., and Trumper, M., *Clinical Biochemistry* (ed. 3), London, Saunders, 1945.
45. Cantarow, A., and Trumper, M., *Clinical Biochemistry* (ed. 4), London, Saunders, 1949.
46. Cantarow, A., and Trumper, M., *Clinical Biochemistry* (ed. 5), London, Saunders, 1955.
47. Carter, W. J., Hoerman, K. C., Englander, H. R., and Shklair, I. L., *Science* **123**, 325 (1956).
48. Charney, J., and Tomarelli, R. H., *J. Biol. Chem.* **140**, 711 (1947).
49. Cherry, I. S., and Crandall, L. A., *Amer. J. Physiol.* **100**, 266 (1932).
50. Chinsky, M., Shmagranoff, G. L., and Sherry, S., *J. Lab. Clin. Med.* **47**, 108 (1956).
51. Cohen, P. P., and Hekhuis, G. L., *J. Biol. Chem.* **140**, 711 (1941).
52. Cram, D. M., and Rossiter, R. J., *Biochem. J.*, **43**, 21 (1948).
53. Cumings, J. N., Goodwin, H. J., and Earl, C. J., *J. Clin. Path.* **8**, 69 (1955).
54. Dalgaard, J. B., *Acta Physiol. Scand.* **13**, 310 (1947).
55. Dalgaard, J. B., *Acta Physiol. Scand.* **16**, 287 (1949).
56. Daniel, O., and Van Zyl, J. J., *Lancet* **i**, 998 (1952).
57. Daniel, O., Kind, P. R. N., and King, E. J., *Brit. Med. J.* **1**, 19 (1954).
58. Danilewsky, A., *Arch. Path. Anat. und Physiol.* **25**, 279 (1862).
59. Demuth, F., *Biochem. Z.* **1599**, 415 (1925).
60. DiStefano, V., Neuman, W. F., Rouser, G., *Arch. Biochem. Biophys.* **47**, 218 (1953).
61. Engel, M. B., and Furuta, W., *Proc. Soc. Exp. Biol. N. Y.* **50**, 5 (1942).
62. Fell, H. B., and Robison, R., *Biochem. J.* **23**, 767 (1929).
63. Fell, H. B., and Robison, R., *Biochem. J.* **24**, 1905 (1930).
64. Fell, H. B., and Robison, R., *Biochem. J.* **28**, 2243 (1934).
65. Fishman, W. H., and Lerner, F., *J. Biol. Chem.* **200**, 89 (1953).
66. Fishman, W. H., Bonner, C. D., and Homburger, F., *Proc. Amer. Asso. Cancer Res.* **1**, 14 (1954).
67. Fleisher, G. A., *J. Clin. Invest.* **32**, 674 (1953).
68. Fleisher, G. A., *J. Biol. Chem.* **206**, 637 (1954).
69. Fleisher, G. A., *Arch. Biochem. Biophys.* **61**, 119 (1956).
70. Fleisher, J. H., and Pope, E. J., *Arch. Indust. Hyg. Occup. Med.*, **9**, 323 (1954).
71. Folin, O., and Ciocalteu, V., *J. Biol. Chem.* **73**, 627 (1927).
72. Franseen, C. C., Simmons, C. C., and McLean, R., *Surg. Gynec. Obstet.* **68**, 1038.
73. Glock, G. E., Pincus, P., and Murray, M. M., *Biochem. J.* **32**, 2096 (1938).
74. Goldblatt, M. W., *Pharm. J.* **164**, 229 (1950).
75. Gray, J. D., and Carter, F. S., *Arch. Dis. Childh.* **24**, 189 (1949).
76. Green, C. H., Shattuck, H. F., and Kaplowitz, L., *J. Clin. Invest.* **13**, 1079 (1934).
77. Gutman, A. B., and Gutman, E. B., *J. Clin. Invest.* **17**, 473 (1938).
78. Gutman, A. B., and Gutman, E. B., *Proc. Soc. Exp. Biol., N. Y.* **48**, 687 (1941).
79. Gutman, A. B., Hogg, B. M., and Olson, K., *Proc. Soc. Exp. Biol. N. Y.* **44**, 613 (1940).
80. Gutman, A. B., Olson, K. B., Gutman, E. B., and Flood, C. A., *J. Clin. Invest.* **19**, 129 (1940).
81. Gutman, E. B., and Gutman, A. B., *J. Biol. Chem.* **136**, 201 (1940).
82. Harden, A., and Robison, R., *Proc. Chem. Soc.* **30**, 16 (1914).
83. Harden, A., and Young, W. J., *J. Physiol.* **32**, Proc. of 12th Nov., 1904 (1905).
84. Harris, R. C., and Olmo, C., *J. Clin. Invest.* **33**, 1204 (1954).
85. Henley, J. S., and Pollard, H. M., *J. Lab. Clin. Med.* **46**, 785 (1955).
86. Herbert, F. K., *Brit. J. Exp. Path.* **16**, 365 (1935).
87. Herbert, F. K., *Biochem. J.* **38**, 23 (1944).
88. Herbert, F. K., *Quart. J. Med.* **15**, 221 (1946).
89. Hestrin, S., *J. Biol. Chem.* **180**, 240 (1949).

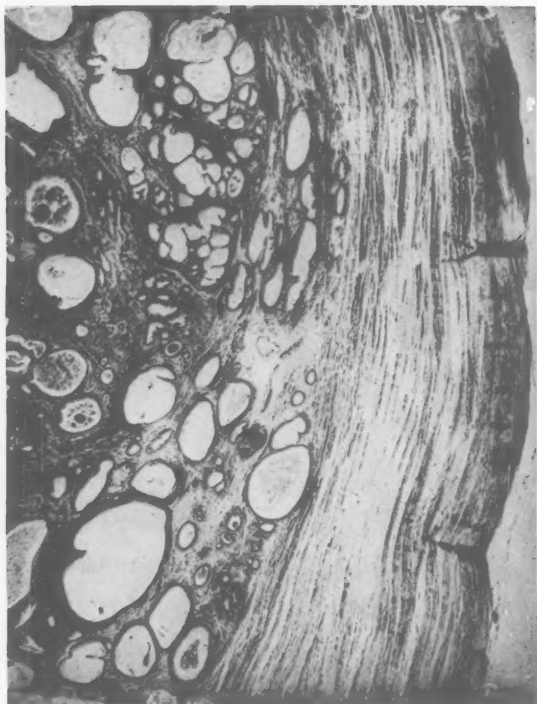


90. Hill, J. H., *Amer. J. Clin. Path.* **26**, 120 (1956).
91. Hock, E., and Tessier, R. N., *J. Urol.* **62**, 488 (1949).
92. Holmberg, C. G., and Laurell, C.-B., *Acta Chir. Scand.* **2**, 550 (1948).
93. Howes, E. L., *Surg. Clin. N. Amer.* 497 (1956).
94. Hudson, R. B., Tsuboi, K. K., and Mittelman, A., *Amer. J. Med.* **19**, 895 (1955).
95. Huggins, C. B., *Biochem. J.* **25**, 728 (1931).
96. Huggins, C. B., and Talalay, P., *J. Biol. Chem.* **159**, 399 (1945).
97. Huggins, C. B., Stevens, R. E., and Hodges, C. V., *Arch. Surg.*, Chicago, **43**, 209 (1941).
98. Jackson, S. H., *J. Biol. Chem.* **198**, 553 (1952).
99. Jaffe, H. L., and Bodansky, O., *Bull. N. Y. Acad. Med.* **19**, 831 (1943).
100. Janowitz, H. D., *Amer. J. Med.* **13**, 465 (1952).
- 100a. Jenner, H. D., and Kay, H. D., *Brit. J. Exp. Path.* **13**, 22 (1932).
101. Johnson, T. A., and Boekus, H. L., *Arch. Intern. Med.* **66**, 62 (1940).
102. Kahle, P. J., Ogden, H. D., and Getzoff, P. L., *J. Urol., Balt.* **48**, 83 (1942).
103. Karmen, A., Wroblewski, F., and La Due, J. S., *J. Clin. Invest.* **34**, 126 (1955).
104. Kattus, A. A., Watanabe, R., Semenson, C., Drell, W., and Agress, C., *J. Amer. Med. Asso.* **160**, 16 (1956).
105. Kay, H. D., *Brit. J. Exp. Path.* **10**, 253 (1929).
106. Kay, H. D., *J. Biol. Chem.* **89**, 235 (1930).
107. King, E. J., *J. Soc. Chem. Ind.*, Lond. **57**, 85 (1938).
108. King, E. J., *Micro-analysis in Medical Biochemistry*, London, Churchill, 1945.
109. King, E. J., and Armstrong, A. R., *Canad. Med. Asso. J.* **31**, 376 (1934).
110. King, E. J., and Delory, G., *Biochem. J.* **33**, 1185 (1939).
111. King, E. J., and Delory, G., *Biochem. J.* **37**, 547 (1943).
112. King, E. J., and Wootton, I. D. P., *Micro-analysis in Medical Biochemistry* (ed. 3), London, Churchill, 1956.
113. King, E. J., and Delory, G., *Post. Grad. Med. J.* **24**, 299 (1948).
114. King, E. J., Wood, E. J., and Delory, G. E., *Biochem. J.* **39**, 24 (1945).
115. La Due, J. S., Wroblewski, F., and Karmen, A., *Science* **120** 497 (1954).
116. Lawford, F. H., The enzymatic hydrolysis of phosphoric esters. Thesis for Ph.D. degree of University of Toronto. (1937).
117. Lesny, I., and Polacek, L., *Acta Neurol. Psychiat. Belg.* **51**, 601 (1951).
118. Levy, A. H., and Levine, S., *Gastroenterology* **30**, 270 (1956).
119. Liebig, J. V., and Wöhler, *Ann. Chem.* **22**, 1 (1837).
120. Lura, H. E., *J. Dental Research* **26**, 203 (1947).
121. McArdle, B., *Quart. J. Med.* **9**, 107 (1940).
122. McCorkle, H., and Goldman, Leon, *Surg. Gynec. Obstet.* **74**, 439 (1942).
123. MacDonald, W. E., Jr., Pollard, C. B., and Gropp, A. H., *A.M.A. Arch. Indust. Hyg.* **6**, 271 (1952).
124. McKelvie, A. M., and Mann, F. C., *Proc. Mayo Clin.* **23**, 449 (1948).
125. MacLagan, N. F., *Brit. Med. J.* **2**, 363 (1944).
126. Marchand, J. F., *J. Amer. Med. Asso.* **149**, 738 (1952).
127. Martland, M., and Robison, R., *Biochem. J.* **20**, 847 (1926).
128. Mendel, B., and Rudney, H., *Biochem. J.* **37**, 59 (1943).
129. Meranze, T., Meranze, D. R., and Rothman, M. M., *Rev. Gastroenterol.* **6**, 254 (1939).
130. Meyer, L. M., Sawitsky, A., Ritz, N. D., and Fitch, H. M., *J. Lab. Clin. Med.* **33**, 189 (1938).
131. Michel, H. O., *J. Lab. Clin. Med.* **34**, 1564 (1949).
132. Milles, H. L., and Salt, H. B., *Brit. Med. J.* **2**, 444 (1950).
- 132a. Morris, N., and Peden, O. D., *Quart. J. Med.* **6**, 211 (1937).

133. Necheles, H., Meyer, J., Bridgwater, A. B., Sorter, H., and Wulkan, E., *J. Appl. Physiol.* **3**, 559 (1956).
134. Neuman, W. F., and Neuman, M., *Chem. Rev.* **53**, 1 (1953).
135. Nothman, M. M., Pratt, T. D., and Benotti, J., *J. Lab. Clin. Med.* **33**, 833 (1948).
136. Ohmori, Y., *Enzymologia* **4**, 217 (1937).
137. Patwardhan, V. N., Chitre, R. G., and Sukhatankar, D. R., *Indian J. Med. Res.* **32**, 31 (1944).
138. Payne, W. W., *Great Ormond St. J.* **3**, 23 (London). (1952).
139. Peden, O. D., *Arch. Dis. Childh.* **12**, 87 (1937).
140. Pritchard, J. A., and Weisman, R., *J. Lab. Clin. Med.* **47**, 98 (1956).
141. Rae, J. J., *J. Dental Research* **20**, 453 (1941).
142. Ravin, H. A., *Lancet* **726**, (1956).
143. Ravin, H. A., and Seligman, A. M., *Arch. Biochem. Biophys.* **42**, 337 (1953).
144. Reiner, Miriam, *Standard Methods of Clinical Pathology*, New York, Acad. Press, 1953.
145. Reinhold, J. G., Tourigny, L. G., and Yonan, V. L., *Amer. J. Clin. Path.*, **23**, 645 (1953).
146. Reynolds, O. B., Reynolds, M. D., and Walker, B. S., *Clin. Chem.*, **2**, 117 (1956).
147. Richards, C. H., and Wolff, H. G., *Arch. Neurol. Psychiat.* **43**, 59 (1940).
148. Roberts, W. M., *Brit. Med. J.* **1**, 734 (1933).
149. Robison, R., *Biochem. J.*, **16**, 809 (1922).
150. Robison, R., and Rosenheim, A. H., *Biochem. J.*, **28**, 684 (1934).
151. Roche, J., *Biochem. J.* **25**, 1724 (1931).
152. Roche, J., *Ann. Nutr. Paris* **1**, 3 (1947).
153. Roche, J., *The Enzymes: Chemistry and Mechanism of Action* (Sumner, J. B., and Myrback, K. D. R., Eds.,) New York, Acad. Press, 1950, vol. I., pt. I., p. 503.
154. Roche, J., and Bullinger, E., *Bull. Soc. Chim. Biol., Paris* **21**, 166 (1939).
155. Roche, J., Thoai, N.-v., and Baudoin, J., *Bull. Soc. Chim. Biol., Paris* **24**, 247 (1942).
156. Roche, J., Thoai, N.-v. and Roger, M., *Bull. Soc. Chim. Biol., Paris* **26**, 1047 (1944).
157. Rudolph, L. A., Dutton, R., and Schaefer, J. A., *J. Clin. Invest.* **34**, 960 (1955).
158. Sabine, J. C., *J. Clin. Invest.* **19**, 833 (1940).
159. Sabine, J. C., *Blood* **6**, 151 (1951).
160. Schapira, G., Dreyfus, J. C., and Schapira, F., *Sem. hôp. Paris* **29**, 1917 (1953).
161. Scheinberg, I. H., and Gitlin, D., *Science* **116**, 484 (1952).
162. Seudamore, H. H., Vorhaus, L. J., and Kark, R. M., *Blood* **6**, 1260 (1951).
163. Seligman, A. M., and Nachlas, M. M., *J. Clin. Invest.* **29**, 31 (1950).
164. Seligman, A. M., Chauncey, H. H., Nachlas, M. M., Mannheimer, L. H., and Ravin, H. A., *J. Biol. Chem.* **190**, 7 (1951).
165. Shibata, S., and Takahashi, H., **3**, 188 (1953).
166. Shinowara, G. Y., Jones, L. M., and Reinhart, H. L., *J. Biol. Chem.* **142**, 921 (1942).
167. Sibley, J. A., and Lehninger, A. L., *J. Nat. Cancer Inst.* **9**, 303 (1949a).
168. Sibley, J. A., and Lehninger, A. L., *J. Biol. Chem.* **177**, 859 (1949b).
169. Sibley, J. A., Higgins, G. M., and Fleisher, G. A., *Arch. Path.* **59**, 712 (1955).
170. Siffert, R. S., *J. Exp. Med.* **93**, 415 (1951).
171. Somogyi, M., *Arch. Intern. Med.* **67**, 665 (1941).
172. Stedman, E., Stedman, Ellen, and Easson, L. H., *Biochem. J.* **26**, 2056 (1932).
173. Stern, M. I., *Brit. J. Nutr.* **1**, 182 (1948).
174. Stewart, C. B., Sweetser, T. H., and Delory, G. E., *J. Urol.* **63**, 128 (1950).
175. Sullivan, I. J., Gutman, E. B., and Gutman, A. B., *J. Urol. Balt.* **43**, 426.
176. Sunderman, F. W., *Amer. J. Clin. Path.* **12**, 404 (1942).
177. Swan, K. C., and Myers, H. B., *Arch. Neurol. Psychiat.* **38**, 288 (1937).
178. Swanson, M. A., *J. Biol. Chem.* **184**, 647 (1950).



**Fig. 1.** Deposition of bone salts in epiphyseal cartilage, by action of phosphatase on phosphoric ester. *Upper left:* Tibia of a rachitic rat. *Lower left:* Same tibia (serial slice) immersed in solution of phosphoric ester (calcium glycerophosphate). Note extensive calcification. *Upper right:* Humerous of rachitic rat immersed in solution of inorganic calcium phosphate. Note slight calcification. *Lower right:* Tibia immersed in solution of phosphoric ester (calcium glycerophosphate). Note extensive calcification (after Robison, 1932).



**Fig. 5.** Prostate gland stained for acid phosphatase (Gomori's method), and showing cystic cavities which were filled with prostatic plasma of high acid phosphatase activity (6000 units/ml.). From Daniel and Van Zyl (1952).

179. Swanson, M. A., and Cori, C. F., *J. Biol. Chem.* **172**, 815 (1948).
180. Tagnon, H. J., *Practitioner* **174**, 95 (1955).
181. Taylor, J. F., Green, A. H., and Cori, G. T., *J. Biol. Chem.* **173**, 591 (1948).
182. Tonhazy, N. E., White, N. G., and Umbreit, W. W., *Arch. Biochem.* **28**, 36 (1950).
183. Uzman, L., Denny-Brown, D., *Amer. J. Med. Sci.* **215**, 599 (1948).
184. Vallee, B. L., *Metabolism* **1**, 420 (1952).
185. Volk, B. W., Losner, S., Oronson, S. M., and Lew, H., *Amer. J. Med. Sci.* **232**, 38 (1956).
186. Vorhaus, L. J., Kark, R. M., *Amer. J. Med.* **14**, 707 (1953).
187. Vorhaus, L. J., Seudamore, H. H., and Kark, R. M., *Gastroenterology* **15**, 304 (1950).
188. Wachstein, M., *J. Lab. Clin. Med.* **31**, 1 (1946).
189. Wallman, I. S., and Vidor, G. I., *Lancet* **i**, 1105 (1955).
190. Warburg, O., and Christian, W., *Biochem. Z.* **314**, 399 (1943).
191. Watkinson, J. M., Delory, G. E., King, E. J., and Haddow, A., *Brit. Med. J.* **2**, 492 (1944).
192. White, J. W., *Brit. Med. J.* **2**, 575 (1893).
193. Whitmore, W. F., and Woodward, H. Q., *J. Urol.* **74**, 809 (1955).
194. Wiltshaw, E., and Moloney, W. C., *Blood* **10**, 1120 (1955).
195. Young, J., King, E. J., Wood, E., and Wootton, I. D. P., *J. Obstet. Gynaec. Brit. Emp.* **53**, 251 (1946).

# Mucoprotein Estimation in Clinical Chemistry

Noel F. MacLagan

THE FIRST DETECTION OF MUCOID SUBSTANCES in blood has been ascribed to Freund (1892), although it appears from his description that the substance which he isolated, being free from nitrogen, would now be classed as a mucopolysaccharide rather than a mucoprotein. The earlier work on the mucous secretions, hyaluronic acid, and chondroitin sulfate, is also remote from our present subject, and the first work on mucoproteins of clinical significance was probably that of Katzman and Doisy (1932) on the isolation of gonadotrophins from urine, since these substances are now recognized as being mucoproteins. Later studies by Rimington and Rowlands in 1941 showed that the same class of substances could be isolated from serum.

In 1933, Brdicka demonstrated the existence of substances with unusual polarographic properties, both in cancer serum and in its protein-free filtrate. Ten years later, this problem was reinvestigated by Winzler and Burk (1943), who demonstrated that the substance responsible was mucoprotein in nature. More recently, interest in the urinary mucoprotein has been revived following work by Tamm and Horsfall (1950), who showed that normal urine contained a mucoprotein with viral antihemagglutin properties. There has also been a revival of interest in the polarographic technic, to which new methods have been applied by Balle-Helaers (1956).

The development of the subject during the last decade has owed much to the work of Meyer (1945) on the classification of this group of substances, and his views on the nature of protein-carbohydrate complexes is illustrated in Fig. 1. The broad distinction which grew up between those containing more than 4 per cent of hexosamine, which he called mucoids, and glycoproteins containing less than 4 per cent of hexosamine has been of great orientating value. The term "mucoid" was, however, still used to cover rather a wide range of substances, and the further subdivision of this group proposed by Morgan (1953) is a valuable addition. As will be seen from the diagram, Morgan subdivided the mucoid group into mucoids proper containing carbohy-

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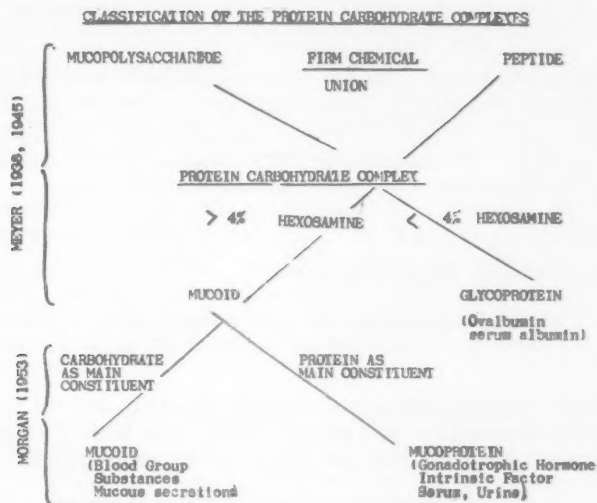


Figure 1

drates as the main constituent, and mucoproteins which contain a preponderance of protein. The latter group, to which the present paper will be restricted, contain substances of great biologic importance such as the gonadotrophic hormones, the intrinsic factor of Castle, and the heterogenous group of mucoproteins found in blood, urine, semen, etc. As clinical chemists, our interest at present is practically confined to the estimation of these substances in serum and in urine.

### ESTIMATION OF SERUM MUCOPROTEINS

The principal chemical methods of estimation are summarized in Table 1. It will be seen from this that many workers have adopted a preliminary precipitation of heat-coagulable protein either with perchloric acid, trichloroacetic acid, or sulfosalicylic acid. The mucoprotein itself may then be estimated in the filtrate either by direct colorimetry, or by a second precipitation with 5 per cent phosphotungstic acid in 2N HCl, followed by some suitable colorimetric, turbidimetric, or chemical estimation. The methods of Seibert (1946), Shetlar (1948), and Ayala, Moore, and Hess (1951), differ from the others mentioned since they do not exclude the carbohydrate component of the heat coagulable protein and, therefore, give higher values than the other three methods mentioned. Most of the methods have employed different arbitrary standards such as single proteins, sugars, or amino acids, and it is, therefore, difficult to compare the results directly. The method of Anderson and Mac-

Table 1. SERUM MUCOPROTEINS: CHEMICAL METHOD OF ESTIMATION

Worker	Precipitation of heat-coagulable protein	Precipitation of mucoprotein	Estimation of mucoprotein
Winzler	Perchloric acid	5% Phosphotungstic acid in 2N HCl	Biuret, orcinol, phenol, digestion
de la Huerca <i>et al.</i> (1956)	Perchloric acid	5% Phosphotungstic acid in 2N HCl	Turbidimetric
Seibert (1946)		Ethanol	Carbazole biuret
Shetlar (1948)		Sodium sulfate, ethanol	Tryptophane biuret
Ayala, Moore, & Hess (1951)	Trichloroacetic acid		Diphenylamine reaction
Anderson, & MacLagan (1954)	Sulfosalicylic acid	5% Phosphotungstic in 2N HCl	Diphenylamine reaction

lagan (1954) employs a urinary mucoprotein standard, and the results can therefore be expressed in more absolute terms. This method gives a mean normal value of  $136.5 \pm 3.9$  mg. per 100 ml. in 54 normal subjects (S.D. 29).

### ELECTROPHORESIS OF SERUM MUCOPROTEIN

At the conventional value of pH 8.5, the serum mucoproteins migrate with the alpha-globulins and are not distinguishable from the other alpha-globulins except by special staining methods such as the periodic acid-Schiff stain (Winzler, Devor, and Mehl, 1946). At pH 4-4.5, however, the mucoproteins reveal their unusually low isoelectric point by showing a considerable migration to the anode, while all the other serum proteins remain stationary or migrate to the cathode (Peterman, Karnovsky, and Hogness, 1948; Boyland, Butler, and Conway, 1951). In this way, fractions  $M_1$  and  $M_2$  were distinguished by Winzler, the former having a greater mobility than the latter. The advent of paper electrophoresis has permitted the application of special carbohydrate stains, such as the periodic acid-Schiff stain, which has greatly facilitated the identification of mucoprotein complexes in serum. The most elegant development of this is the recent work of Markham (1956), in which the electrophoresis is made in two directions at right angles, first at pH 8.6, and later at pH 4.5. This method is a considerable aid to the separation of the serum mucoproteins.

### METHODS OF ISOLATING URINE MUCOPROTEINS

The earlier methods of isolating mucoproteins from urine were not suitable as methods of quantitative analysis. These included the use of permittit or benzoic acid adsorption by Katzman and Doisy (1932), precipitation with 0.58M NaCl by Tamm and Horsfall, and preliminary concentration followed by alcohol precipitation (Waldron, 1952).



A quantitative method for the estimation of one of the many fractions has been proposed by Anderson and MacLagan (1955). This employs a preliminary adsorption with benzoic acid as in the Katzman and Doisy method. The benzoic acid is removed with acetone and the mucoprotein is partially purified by solution in water and reprecipitation with acetone. It is finally estimated by the diphenylamine reagent of Ayala, Moore, and Hess (1951). This acid solution of diphenylamine is an extremely useful reagent. It was originally employed by Jolles (1910) for the demonstration of fructose, and has also been used by Dische (1929). It was shown by Niazi and State (1948) to give color with mucoproteins, and it appears from the work of Blix (1952) and Odin (1952) that the sialic acid present in combined form in the mucoprotein is the substance which actually gives rise to the color.

By this method normal subjects were found to secrete  $134.5 \pm 6.1$  mg. per 24 hours (S.D. 34).

#### RENAL CLEARANCE OF MUCOPROTEIN

An important development of this quantitative study of the urine mucoproteins described above has been the suggestion that the substance estimated in this way probably originates mainly from the serum. This is suggested by the strong positive correlation between values in serum and urine, shown in Fig. 2. It is possible from these results to calculate the approximate renal clearance for the mucoproteins and values for this are given in Table 2, where

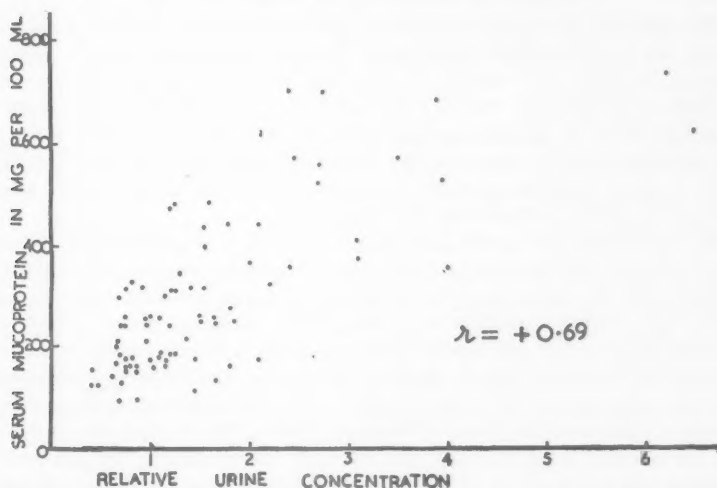


Fig. 2. Serum and urine mucoproteins in 80 cases of cancer. (From Loekey, Anderson, and MacLagan, *Brit. J. Cancer*, 1956).

Table 2. NORMAL RENAL CLEARANCE OF PROTEINS

Protein	Average plasma level (mg. per 100 ml.)	Average urinary excretion (mg. per 24 hr.)	Apparent clearance (ml. per 24 hr.)
Albumin	4000	15*	0.4
Globulin	2000	26*	1.3
Mucoprotein			
Normal	137	135	99
Cancer	308	238	77
Endocrine	144	197	137

\*From data of Rigas and Heller (1951).

they are compared with the renal clearance of other plasma proteins. It will be seen from this that the apparent renal clearance is enormously greater for mucoproteins, as compared with albumin and globulin. This gross difference presumably correlates with the molecular weight of the fraction concerned, which is of the order of 20,000, and possibly with its different molecular shape. It should be stressed here that the fraction estimated by the method of Anderson and MacLagan (1955) appears to be quite different from that isolated by Tamm and Horsfall (1950). The latter had a molecular weight of  $7 \times 10^6$  and was thought to originate in the urinary tract.

### BIOLOGIC PROPERTIES OF MUCOPROTEIN

The mucoproteins as a class exhibit a variety of biologic properties, many of which are of physiologic importance. Positive properties include gonadotrophic, thyroxine and vitamin B<sub>12</sub> binding, amylase activity, and influence on leukocyte migration. On the other hand, these substances are inhibitors to viral agglutination, blood coagulation, trypsin activity, and a wide range of precipitation reaction, including the flocculation tests of liver function. This wide range of negative properties suggests a possible importance of mucoproteins as protective colloids in the body.

This protective or "antiprecipitation" function is of considerable interest in at least two different connections. Firstly, a relationship between urinary colloids and the tendency to the formation of urinary calculi has been suspected by a number of workers (e.g., Butt, 1955). Although there is at present no clear evidence that mucoproteins are concerned in this, the question appears to merit further investigation. Secondly, a negative correlation has been noted between serum mucoprotein concentration and certain flocculation tests of liver function (Anderson, Lockey, and MacLagan, 1955). The thymol and gold tests are particularly implicated and the addition of mucoprotein to sera giving positive tests can be shown to inhibit these reactions. The serum mucoproteins must therefore be credited with a significant influence on these flocculation tests.

## URINE AND SERUM MUCOPROTEINS IN DISEASE

Variations of serum mucoproteins in disease have been the subject of a recent review by Greenspan (1955), who has himself contributed a great deal to this subject. Broadly speaking, high values are encountered in cancer, in collagen diseases, and in many acute and chronic infections. Less striking elevations have been reported in obstructive jaundice. Low values are found in hepatitis, in various endocrine diseases, and in nephrosis and myelomatosis. In most of these conditions, although a high proportion of abnormal results are found, there is considerable overlap with the normal range. Nevertheless, in jaundice the difference between the results in hepatitis and obstructive jaundice is very striking, and the estimation is of definite diagnostic value in special cases.

In our work at the Westminster Medical School, we have concentrated mainly on the urine mucoprotein estimation with special reference to cancer, and some of our results are shown in Figs. 3, 4, and 5. In Fig. 3 is shown the marked positive correlation which we found between urine mucoprotein concentration and urine specific gravity. This relationship suggested the use of a function which we have called the relative urine concentration (R.U.C.) when comparing one group of patients with another. This function is obtained by dividing the urine mucoprotein concentration in mg. per 100 ml. by the excess of specific gravity over 1000. Mean values for this quantity were  $0.60 \pm 0.024$  in 29 normal subjects (S.D. 0.13).

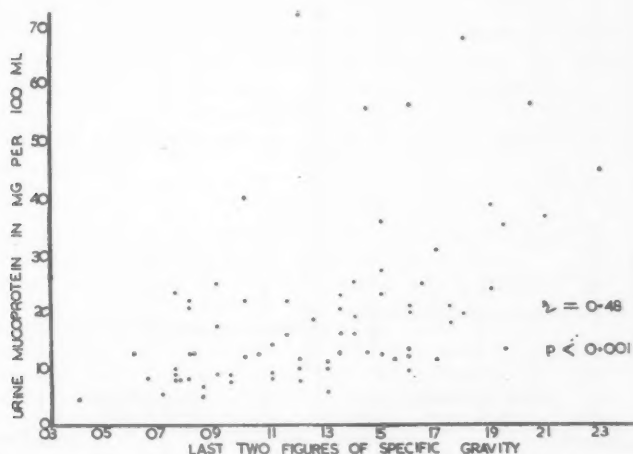


Fig. 3. Correlation between urine mucoprotein concentration and specific gravity in 75 cases of cancer. (From Loekey, Anderson, and MacLagan, *Brit. J. Cancer*, 1956.)

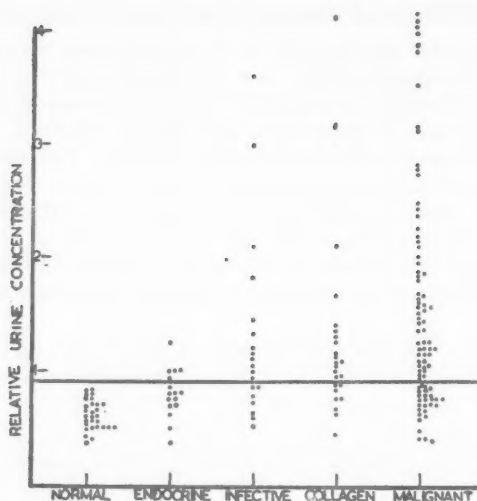


Fig. 4. Urine mucoproteins (R.U.C.) in cancer and other conditions. (From Lockey, Anderson, and MacLagan, *Brit. J. Cancer*, 1956.)

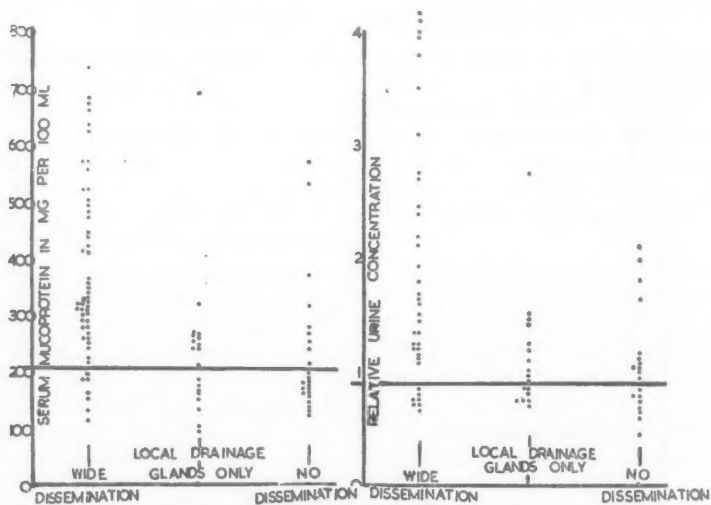


Fig. 5. Urine and serum mucoproteins in cancer in relation to degree of dissemination. (From Lockey, Anderson, and MacLagan, *Brit. J. Cancer*, 1956.)

Figure 4 shows the general distribution of results in cancer and in some other conditions. It will be seen that while there were high values in the malignant group, there was overlap with the normal range. Figure 5 shows the distribution of values in cancer in relation to the degree of dissemination. It will be seen that both the serum and urine values show some degree of correlation with the degree of spread, although there were important exceptions. The low proportion of positive results seen in the localized tumors does not suggest that the test could be used in the early diagnosis of cancer. In cancer, high results were particularly striking in the lymphomas, while in malignant disease confined to bone very little elevation was found; other cancers occupy an intermediate position. The effect of surgical and radiotherapeutic treatment was studied in a small series of patients. Operations almost always produced a large rise, while the results of radiotherapy were more variable, with some suggestion that those cases with a favorable outcome showed a greater fall of mucoprotein values after treatment.

### CONCLUSION

It is difficult to sum up one's impression of the values of mucoprotein estimation in a few words. Except in the case of jaundice, it should perhaps be admitted that at the present time the results are of more academic than practical interest. Nevertheless, it seems that the changes recorded represent a very fundamental type of biologic response which may well have more importance when it is more completely understood. One of the principle obscurities at the present time is the site of origin of these substances in the body. If we could only find out exactly where they were being manufactured, the interpretation of pathologic variations might well become much easier. The difficulties of estimating mucoproteins in tissue extracts are unfortunately considerably greater than is the case with serum or urine, but work on these lines is in progress in my department and we hope to be able to report on this question at some future date.

### REFERENCES

1. Ayala, W., Moore, L. V., and Hess, E. L., *J. Clin. Invest.* **30**, 781 (1951).
2. Anderson, A. J., and MacLagan, N. F., *J. Physiol.* **125**, 44 (1954).
3. Anderson, A. J., and MacLagan, N. F., *Biochem. J.* **59**, 638 (1955).
4. Anderson, Lockey, and MacLagan, *Biochem. J.* **60**, xli (1955).
5. Balle-Helaers, E., *Bruzelles Medical* **36**, 339, 402 (1956).
6. Blix, G., Svennerholm, L., and Werner, I., *Acta Chem. Scand.* **6**, 358 (1952).
7. Boyland, E., Butler, L. D., and Conway, B. E., *Brit. J. Cancer* **5**, 235 (1951).
8. Brdicka, R., *Coll. Czech. Chem. Commun.* **5**, 112 (1933).
9. Butt, A. J., *Advances in Int. Med.* **7**, 11 (1955).
- 9a. de la Hueraga, J., Dubin, A., Kushner, D. S., Dyniewicz, H. A., and Popper, H., *J. Lab. Clin. Med.* **47**, 403 (1956).
- 9b. Dische, Z., *Mikrochemie* **1**, 33 (1929).
10. Freund, E., *Zentralbl. Physiol.* **6**, 345 (1892).

11. Greenspan, E. M., *Advances in Int. Med.* **7**, 101 (1955).
12. Jolles, A., *Munch. Med. Wschr.* **57**, 353 (1910).
13. Katzman, P. A., and Doisy, E. A., *Biol. Chem.* **98**, 739 (1932).
14. Loockey, E., Anderson, A. J., and MacLagan, N. F., *Brit. J. Cancer* **10**, 209 (1956).
15. Markham, R. L., *Nature* **177**, 125 (1956).
16. Meyer, K., *Advances in Protein Chemistry* **2**, 161 (1945).
17. Morgan, W. T. J., *Proc. Roy. Soc. Med.* **46**, 783 (1953).
18. Niazi, S., and State, D., *Cancer Res.* **8**, 653 (1948).
19. Odin, L., *Nature* **170**, 663 (1952).
20. Peterman, M. L., Karnovsky, D. A., and Hogness, K. R., *Cancer* **1**, 104 (1948).
- 20a. Rigas, D. A., and Heller, C. G., *J. Clin. Invest.* **30**, 853 (1951).
21. Rimington, C., and Rowlands, I. W., *Biochem. J.* **35**, 736 (1941).
22. Seibert, F. B., and Atno, J., *J. Biol. Chem.* **163**, 511 (1946).
23. Shetlar, M. R., Foster, J. V., and Everett, M. R., *Proc. Soc. Exp. Biol.* **67**, 125 (1948).
24. Tamm, I., and Horsfall, F. L., *Proc. Soc. Exp. Biol.* **74**, 108 (1950).
25. Waldron, D. M., *Nature* **170**, 461 (1952).
26. Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.* **4**, 417 (1943-44).
27. Winzler, R. J., Devor, A. W., and Mehl, J. W., *Cancer Res.* **6**, 496 (1946).

# Mucoproteins in Clinical Chemistry

Z. Sary

IT HAS BEEN KNOWN FOR MANY YEARS that human and animal tissues contain, besides glucose and glycogen, relatively large amounts (1) of other carbohydrates, whose metabolic paths and biologic functions widely differ from those of glucose and glycogen. These carbohydrates are not easily catabolized energy-producing substances like glucose and glycogen, but relatively stable, nitrogen-containing polysaccharides, forming a part of the tissue structure. They are designed as mucopolysaccharides.<sup>1</sup>

While glycogen deposits accumulate in the enzymatically active interior of the living cell, most mucopolysaccharides are extracellular substances covering cell surfaces and occurring in large amounts in intercellular spaces. While the amount of glycogen deposits in the cells changes continuously during the course of many physiologic processes, tissue mucopolysaccharides are renewed at a relatively slow rate. The amount and distribution of these substances in the tissues remains rather constant under physiologic conditions and their importance for the maintenance of animal life becomes particularly apparent in disease. Research work of these last years, based on chemical and histochemical methods, has shown that the production of mucopolysaccharides by the living cell and the distribution of these substances in the tissues and body fluids is altered in a great number of pathologic processes. Clinical determinations of these substances or their constituents can give important clues for diagnostic and pathogenetic problems.

## METABOLISM OF MUCOPOLYSACCHARIDES

The metabolism of these nitrogen-containing carbohydrates is closely related to the metabolism of protein and its constituents. Most of the mucopolysaccharides present in the human body are firmly bound to protein molecules. There is no sharp boundary in biology between proteins and polysaccharides:

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<sup>1</sup>Mucopolysaccharides are, according to Meyer (6), polysaccharides containing hexosamine as one component, whether they occur free or whether they are obtained from substances of higher molecular weight.

living cells produce proteins combined with small or large polysaccharide groups as well as polysaccharides bound to small groups of polypeptidic structure. There exists a complete scale of intermediate substances in the animal body between protein and polysaccharides: mucoids consist of mucopolysaccharides bound to relatively small polypeptide groups. Mucoproteins are intermediate compounds containing considerable amounts of both polypeptide and polysaccharidic components; glycoproteins are proteins containing a relatively small percentage of carbohydrate. Fibrinogen (2, 3), casein (4, 5), and other proteins, whose amino acid content had been investigated for more than 50 years, have been shown to contain carbohydrate groups firmly bound to the polypeptide chain. Only a relatively small number of proteins consist entirely of amino acid radicals (Table 1).

All mucopolysaccharides hitherto known contain hexosamines. Glucosamine and galactosamine, both in acetylated form, have been isolated from different mucopolysaccharides by chromatographic methods. In some of them large amounts of glucuronic acid and sulfuric acid radicals are present. Some protein-bound mucopolysaccharides contain hexoses (mannose and galactose) and a methylpentose (fucose).

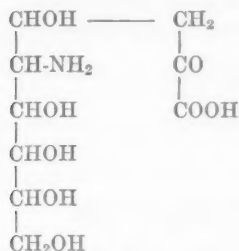
In many mucoproteins the presence of N-containing acids giving the Ehrlich and Bial reactions has been established: neuraminic acid (Klenk, 7-11), sialic acid (Blix, Odin, Werner, 12-15), lactic acid (Kuhn and Brossmer, 16, 17), and serolactic acid (Yamagawa and Suzuki, 18-20) are closely related compounds, deriving from a group of high chemical and biologic activity present in many mucopolysaccharides. The chemical constitution of these N-containing acids is not exactly known at present. While Gottschalk suggests that neuraminic acid may consist of a hexosamine radical bound by aldol condensation to the  $\beta$ -C atom of pyruvic acid, Klenk proposes the constitution of an erythritol ether of an aminoglucuronic acid for the same compound (21).

Table 1. PROTEIN CONTENTS

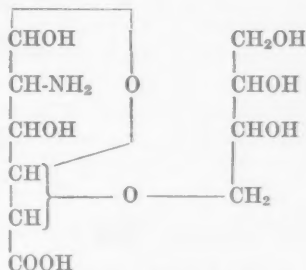
<i>Agluconic proteins containing no polysaccharide groups</i>	<i>Glycoproteins, proteins with small amounts of carbohydrate</i>	<i>Mucoproteins containing large amounts of bound mucopolysaccharide groups</i>	<i>Mucoids consisting of large amounts of mucopolysaccharide bound to relatively small polypeptide groups</i>	<i>Mucopolysaccharides containing no amino acids or polypeptides</i>
Insulin Serum albumin	Globulins Fibrinogen Casein (carbohydrate - content less than 10%)	Mucins Serum mucoproteins (carbohydrate content 10-50%)	Blood group substances (about 85% carbohydrate)	Chondroitin sulfuric acid Hyaluronic acid Heparin



Sialic acid seems to be identical with diacetyl neuraminic acid (22), whereas lactamic acid (16, 17; equal to gynaminic acid, 23, 24) is probably an N-acetyl-neuraminic acid (7). The neuraminic acid-containing unit is split off by some viruses and bacterial enzymes (9, 25, 26).



Neuraminic acid (Gottschalk)



Neuraminic acid (Klenk)

### MUCOPOLYSACCHARIDES IN THE BODY

Mucopolysaccharides contain many more hydrophilic groups than polypeptides or proteins and are able to bind large amounts of water. Some of them have threadlike structure and form hydrated gels and solutions of high viscosity. In animal tissues mucopolysaccharides play a similar role as the pectin substances in plant tissues. Acid mucopolysaccharide gels fill the interfibrillar spaces of connective tissue and there is a high degree of correlation between the water content and the concentration of mucopolysaccharides in connective tissue (27). The cells of parenchymatous organs seem to adhere to each other by thin layers of mucopolysaccharide gels. Polysaccharides containing sulfuric acid radicals are constituents of the intercellular substances of cartilage and bone. Viscous mucoprotein-containing secretions cover the epithelial surfaces of the respiratory, digestive and urogenital organs.

An increase in the mucopolysaccharide content is observed in many tissues under pathologic conditions, and an enhancement of mucopolysaccharide production seems to be a general defense reaction of damaged or irritated cells (28). Irritated nasal or bronchial mucosa produces increased amounts of mucuous secretion. In connective tissues surrounding damaged parts of tissue, the concentration of the interfibrillar hyaluronic acid gels is increased (27). Mucopolysaccharides accumulate in a particularly high concentration in the connective tissue at the periphery of inflammatory lesions caused by bacteria or viruses (28); important elevations of the polysaccharide concentration have been demonstrated also in the connective tissue bordering on tumor transplants in rats (29). Increased mucopolysaccharide production seems to be part of a nonspecific tissue response to various damaging agents, inhibiting the spread of the damaging agent and limiting the damaged area.

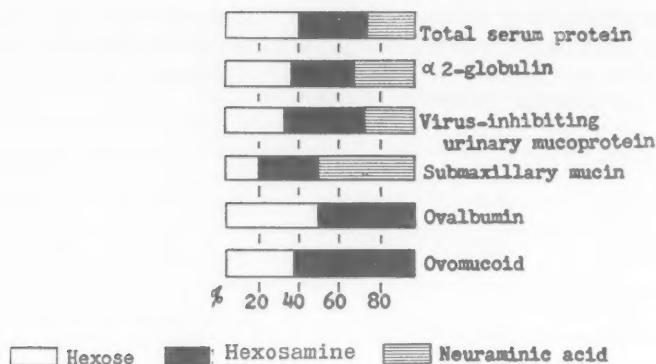


Fig. 1. Distribution of hexose, hexosamine, and neuraminic acid in the carbohydrate groups of various proteins.

#### IN PLASMA

Blood plasma, which forms the intercellular substance of the blood, contains a large number of different mucoproteins and glucoproteins; the total amount of protein-bound carbohydrate present in blood plasma exceeds by far the normal amount of free glucose. The polysaccharide groups of the serum proteins consist chiefly of acetylhexosamine (30), galactose, and mannose (31, 32), probably forming a hexosamine-dihexose complex (33), and neuraminic acid (34). That the hexosamine present in serum protein is *D*-glucosamine has been confirmed by chromatography and other methods (18, 31). In serum mucoprotein the presence of galactosamine has also been demonstrated (36, 37). The presence of fucose has been established in some fractions of serum proteins (35, 38, 39). Glucuronic acid radicals are present only in small amounts (40).

In normal adults the total amount of protein-bound serum carbohydrate corresponds to about 100–110 mg. per 100 ml. hexose (32, 41, 42, 192), 65–80 mg. per 100 ml. glucosamine (41, 42, 192), and 40–65 mg. per 100 ml. neuraminic acid (43). As the hexose:hexosamine ratio is rather constant in the total serum polysaccharide, both hexose and hexosamine determinations in the precipitated serum proteins can be used for the estimation of the total amount of serum polysaccharides.

The mucopolysaccharide level in the blood serum seems to be rather constant under physiologic conditions, occasional fluctuations being chiefly due to changes in the protein content of the serum. Fasting and variations in the carbohydrate or protein content of the diet are of little influence (41). Starvation resulted in lowered polysaccharide levels in rats (44, 45). Small doses of adrenalin or insulin failed to affect the level of protein-bound carbohydrate

(46). In hypoglycemic shock a transitory increase, mainly due to the accompanying hemoconcentration was observed (47). No appreciable changes were observed during the menstrual cycle (41).

Children have lower, and aged individuals slightly higher, serum polysaccharide levels than young adults (41). While the blood serum of the human newborn contains only about 75 per cent of the polysaccharide content of the serum of human adults, the serum of newborn calves and sheep have relatively high amounts of a later-disappearing mucoprotein, called fetuin (48). Fetuin contains about 5.3 per cent hexose, 9.9 per cent hexosamine, and 6 per cent neuraminic acid (49). In rats the mucopolysaccharide level increased from a low value at the age of 4 weeks to a maximum at the age of 11 weeks and showed a slight decrease afterwards (50).

A great variety of pathologic conditions such as infectious diseases, bleedings (51), surgical interventions, burns, etc., caused an increase of the total amount of protein-bound serum polysaccharides. Significant increases were seen in conditions such as tuberculosis (52-55), typhoid fever (56), and other infectious diseases (57-59) in humans and in animals. Inflammatory diseases localized in the pulmonary tissue seem to affect the polysaccharide level to an especially high degree (54, 60); lung tissue contains large amounts of heparin and of other mucopolysaccharides (61). Increases of the polysaccharide level were also found in the serum of patients with rheumatoid arthritis (62, 63, 119), but not in those with degenerative joint disease (64, 65). Acute attacks of gouty arthritis are accompanied by a transitory elevation of the serum polysaccharide level (66). Marked increases are observed in uncontrolled cases of diabetes mellitus (47); in diabetic patients even 1 day of uncontrolled diet can produce a significant hyperpolysaccharidemia (67). In the last months of pregnancy the amount of protein-bound serum polysaccharides rises significantly (68-73). Increases of the total serum mucopolysaccharides occur in malignant diseases (74-78) and in tumor-bearing animals (74, 79-81), whereas no increase has been found in benign tumors (82). Epidermal thermal injury in dogs results in an elevation of the total polysaccharides in both lymph and serum and this increase has been shown to be the result of a simultaneous increase of different fractions of protein-bound polysaccharides (83, 193). In a similar way also, patients with epidermal burns showed an increase in the polysaccharide content of many fractions of serum proteins (84). While increased body temperature produced by external heating was followed by an increase of the carbohydrate content of the  $\alpha$ -globulin fraction, administration of pyrogenic substances increased both the carbohydrate content of the albumin and the  $\alpha$ -globulin fraction (85).

Enhanced production of serum polysaccharides seems to be a part of the general response to stress (45, 86). Elevation of the serum polysaccharide level was observed in dogs following the production of sterile turpentine abscesses, bacterial abscesses, and talc granulomas, and developed also after experimental

surgical operations. These elevations occurred both in the presence and absence of fever. The maximal elevation appeared within 3 to 6 days after the initial injury (87). Significant elevations of the level of protein-bound hexoses were observed in acute and chronic scurvy in guinea pigs (88).

### CARBOHYDRATE IN SERUM POLYSACCHARIDES

In spite of the apparent uniformity with which various pathologic conditions are accompanied by an increase in the total amount of protein-bound serum carbohydrate, significant qualitative differences are observed in hyperpolysaccharidemias of different origin. Using salt fractionation with 22%  $\text{Na}_2\text{SO}_4$  two main fractions of serum polysaccharides can be obtained, each of them containing under normal conditions about 50 per cent of the total amount of protein-bound serum carbohydrates. These two main fractions of serum polysaccharides vary independently under pathologic conditions. While in normal pregnancy the nonprecipitable polysaccharide fraction is elevated (68-71), in cases of toxic pregnancy and in some cases of premature birth (72) a marked increase of the  $\text{Na}_2\text{SO}_4$  precipitable fraction was observed. Most cases of carcinoma (74) and of typhoid fever (56) show an isolated increase of the nonprecipitable fraction (56), while in allergic diseases (allergic asthma, allergic purpura, etc.) chiefly the carbohydrate content of the easily precipitable serum protein fraction is augmented (73, 89). An isolated increase of this fraction was also observed in essential hypertonia (89). While in nonprogressive phases of tuberculosis the level of the nonprecipitable fraction is raised, in allergic and progressive cases an increase in the carbohydrate content of the  $\text{Na}_2\text{SO}_4$ -precipitable globulins is observed (54). In rabbits intoxicated chronically with small amounts of phosphorus a rapid decrease of the  $\text{Na}_2\text{SO}_4$ -soluble fraction was associated with an increase of the  $\text{Na}_2\text{SO}_4$ -precipitable fraction of the serum polysaccharide in the first days, whereas both fractions were diminished in later stages of the intoxication (90).

The carbohydrate content of the electrophoretically separated serum protein fractions has been determined, in 1941, by Blix, Tiselius, and Swensson (91). Determinations of the distribution of protein-bound hexosamine in rat serum proteins fractionized by paper electrophoresis were performed by Boas (92). By the use of paper electrophoresis combined with fuchsin sulfite staining (93) the determination of the relative carbohydrate distribution in serum protein fractions can be performed as a clinical routine method. Although the migration velocity of some mucoprotein fractions in paper electrophoresis is slowed down by adsorption to the surface of the paper strips and results obtained by paper electrophoresis may not always correspond exactly to the carbohydrate distribution in protein fractions obtained in the electrophoretic cell (94), paper electrophoresis gives very important information on the carbohydrate content of the protein fractions in normal and pathologic sera. Photometric evaluation of fuchsin-sulfite stained electrophoresis strips has been found in

good agreement with carbohydrate distribution values obtained by chemical determination of protein-bound hexoses, hexosamine, and sialic acid in protein fractions eluted from the paper strip (95).

Table 2. DISTRIBUTION OF THE LEUCOFUCHSIN-SULFITE POSITIVE MATERIAL

	Albumin $\alpha_1$ -globulin	$\alpha_2$ -globulin	$\beta$ -globulin	$\gamma$ -globulin
Staining method	27.3	36.5	26.8	9.4
Elution method	26.8	36.5	26.7	10.0

To determine the parts taken by the different polysaccharide components in fuchsin-sulfite staining, a number of tests with different mucoproteins containing various amounts of hexose, hexosamine, and neuraminic acid were made by Björnesjö (95). For all these mucoproteins the degree of staining corresponded to the sum of hexoses, hexosamine, and sialic acid. These experiments suggest that the color intensity in the fuchsin-sulfite staining is correlated to the total amount of protein-bound polysaccharide and that the variations in the composition of the prosthetic carbohydrate groups are of less importance for their determination by this method. Although only the relative carbohydrate distribution in serum protein fractions can be determined by the fuchsin-sulfite staining method; absolute polysaccharide values can be obtained when the fuchsin-sulfite staining is completed by the determination of the total amount of protein-bound hexoses in the analyzed serum. The distribution of protein and protein-bound carbohydrate shown in Fig. 2 is based on determinations performed by the use of this method by Arnaki from our institute and is in general agreement with reports of other authors (95, 96).

Determinations of the carbohydrate content of the different electrophoretic serum protein fractions show a wide range of variations in pathologic cases (97). While in some pathologic conditions chiefly the amount of carbohydrate present in the  $\alpha_1$ -globulin is increased, in other cases the carbohydrate content of the  $\alpha_2$ -globulins or of the  $\beta$ -globulin fractions is augmented. Immunization and infection increase the amount of  $\gamma$ -globulins, but have no apparent effect on the percentage carbohydrate content of this fraction (98, 99). Likewise, in cases of kala-azar, in which the  $\gamma$ -globulin level shows a tremendous increase, the  $\gamma$ -globulin carbohydrate increases in a proportion similar to the  $\gamma$ -globulin amount itself (100). On the other hand, in the electropherograms of some cases of malignant disease published by other authors (82, 97) a marked increase of the carbohydrate:polypeptide relation of  $\gamma$ -globulins is evident. The slow migrating protein fraction found in Waldenström macroglobulinemia (184, 185) contains especially great amounts of fuchsin-sulfite positive material and seems to contain a mucoprotein component. In most hyperpolysaccharidemias a simultaneous increase of the carbohydrate content of two or more protein fractions is observed.

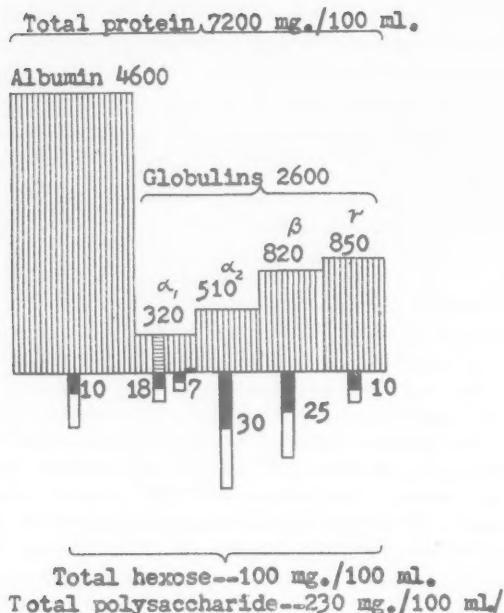


Fig. 2. The average normal distribution of protein-bound carbohydrate on the serum protein fractions obtained by paper electrophoresis. Hatched areas correspond to the polypeptide part of the serum proteins, black areas to their hexose content, and white areas to the hexosamine-neuraminic acid content of the protein fractions. Horizontally hatched area in the  $\alpha_1$ -globulin fraction corresponds to the amount of orosomucoprotein.

#### OROSOMUCOPROTEIN (OROSOMUCOID)

One of the mucoproteins migrating in the  $\alpha_1$ -globulin fraction has been isolated by Weimer, Mehl, and Winzler (101-103) and by Schmid (104, 105). This mucoprotein, called *orosomucoid*,<sup>2</sup> corresponds to about 1 per cent of the total amount of serum protein and contains about 10 per cent of the total amount of protein-bound serum carbohydrate. Orosomucoproteins of different animal species differ in the quantity (106), and in the amount and composition of the carbohydrate groups (107). Serum mucoprotein of normal human subjects contained about 16.5 per cent hexose, 12.0 per cent hexosamine (107), 11.2 per cent (107) and 10 per cent (37) sialic acid, and 63 per cent protein (107). Determinations of the carbohydrate content of serum mucoproteins isolated from the blood in different cases indicate that the carbohydrate content

<sup>2</sup>As the polypeptidic group amounts to 63 per cent, the name *orosomucoprotein* seems to be more adequate.

of this fraction can show a marked increase in disease (64), which is perhaps due to an increased amount of a carbohydrate-rich subfraction (64). In a similar way  $\alpha$ -globulin isolated from the plasma of tumor-bearing mice contained much more carbohydrate than the same fraction obtained from normal controls (80). Clinical determinations of this substance are based on the fractionation by perchloric acid. When serum is treated with perchloric acid, serum proteins (but not orosomucoprotein) are precipitated and orosomucoprotein can be determined in the filtrate. Colorimetric (108) and turbidometric (109) methods of its determination have been developed by Greenspan and others. Orosomucoprotein levels are higher in normal men than in women between the ages of 20 and 50 (109, 110). Slight elevation was observed in aged individuals (111). A diurnal increase, not influenced by meals, occurs in late afternoon (111). Bacterial pyrogen and ACTH elicited mucoprotein increments of 50 per cent, and prompt but transient increases were caused by the administration of parathyroid (111, 112).

Orosomucoprotein levels show typical increases in diseases characterized by inflammatory, degenerative, or traumatic tissue changes (108, 113), and a part of the increases of total amounts of protein-bound serum carbohydrate observed in various diseases is due to the elevated mucoprotein content. In dystrophic infants losses of body weight were strictly associated with increased serum mucoprotein levels (114). In contrast to the marked increases observed in many cases of advanced neoplastic disease (115), multiple myeloma was associated frequently with a reduction in the serum mucoprotein concentration (108). While normal or elevated orosomucoprotein levels are generally associated with various forms of biliary obstruction, reduced concentrations are observed in patients with diffuse hepatocellular diseases (110, 116-118). Depressed orosomucoid values have been also found in patients with pituitary and adrenal insufficiency. Increases in mucoprotein levels as a result of cortisone therapy in Addison's disease or of ACTH therapy in panhypopituitarism have been observed in cases with normal liver function.

#### INCREASED SERUM CARBOHYDRATE

Some of the clinical tests, based on the colloid properties of the blood serum, are influenced by the carbohydrate groups present in the serum proteins. As mucoproteins are not easily coagulated by heating, the thermolability of the serum proteins decreases in hypermucopolysaccharidemia and the Weltman coagulation band is shortened (62, 120). Asymmetrically shaped carbohydrate-containing protein fractions may be one of the components responsible for an increased erythrocyte sedimentation rate. In some diseases (e.g., tuberculosis and rheumatic arthritis) a significant correlation between the erythrocyte sedimentation rate (ESR) and the total serum carbohydrate have been demonstrated (121, 62, 122). In other diseases (as typhoid fever and diabetes mellitus) no correlation has been found between the ESR and the serum poly-



saccharide level (129). Although serum mucoprotein has some anticoagulant activity (123), no correlation of the serum mucoprotein level with hemorrhagic syndromes, reported to be associated with the presence of heparin-like substances in the serum (124), has been observed.

Another factor associated in many cases with an increased level of protein-bound serum carbohydrate is the C-reactive protein, precipitated specifically by the C-polysaccharide of pneumococcus. This factor, which is not present in detectable amounts in normal serum, has been found in the serum in the acute phase of several infectious diseases, acute rheumatic fever, cancer, and myocardial infarctions (125). In some of these states (e.g., tuberculosis, acute rheumatic arthritis, and cancer) the occurrence of the C-reactive protein is significantly correlated with the elevated serum mucopolysaccharide and total serum polysaccharide levels, whereas in pregnancy, in which the amount of protein-bound carbohydrate is increased, the C-reactive protein could be detected only occasionally (75). It is not known at present whether the reaction of this factor with the bacterial C-polysaccharide is mediated by neuraminic acid.

#### VARIOUS FRACTIONS

A relatively small fraction of the protein-bound serum polysaccharide contains glucuronic acid and sulfuric acid radicals; the hexose:hexuronic acid ratio has been found to be about 10:1 in total serum protein (40). Heparin, demonstrable in normal blood serum in amounts of about 0.01 mg. per 100 ml. (126) forms a part of this fraction. A metachromatic polysaccharide fraction, perhaps related to heparin (127) and not identical with any of the serum mucoprotein fractions obtained by other methods (128), forms a constituent of the nonspecific hyaluronidase inhibitor (129). The inhibitor is destroyed by heating at 55° and migrates predominantly with the albumin fraction in an electrophoretic field at pH 8.6 in a barbiturate buffer (130, 131); a smaller part migrates at a slightly slower rate corresponding to the  $\alpha_1$ -globulin fraction (129). The level of the nonspecific hyaluronidase inhibitor is elevated in many of the clinical circumstances and experimental situations in which an increase of serum mucoproteins and protein-bound serum carbohydrate is observed. Significant elevations have been described in acute infections of bacterial and viral origin (132-135), rheumatic fever (136), acute glomerulonephritis (137), nephrotic diseases (138), and in certain skin diseases (139). Low levels of total mucopolysaccharide as well as of hyaluronidase inhibitor were found in the serum of the newborn, while increased levels were demonstrated in the maternal blood (140, 141). On the other hand, no conformity exists in liver disease and high inhibitor levels were found in cases of parenchymatous liver damage in which the mucoprotein content of the serum is usually diminished (142). Neither immunization nor anaphylactic shock in rabbits produced demonstrable changes in the inhibitor level of the serum (143). In some



patients hyaluronidase inhibitor values fluctuated independently of mucoprotein concentration (144).

#### SITE OF ORIGIN OF SERUM MUCOPROTEIN

Although smaller amounts of protein-bound serum carbohydrate may originate in other tissues, the main site of the normal serum mucoprotein production is the liver. While heavy blood losses produce a significant elevation in serum mucopolysaccharide levels in normal rabbits, no increase is observed after bleeding in P-intoxicated rabbits (51). On the contrary, poisons of the bone marrow such as lead or benzene given parenterally do not affect the increase of protein-bound serum polysaccharide induced by bleeding (51). Increases of protein-bound serum carbohydrate occurring regularly in tumor-bearing animals with normal livers, are not observed if the liver of the animals is damaged by specific poisons. Not only serum mucoprotein (145) but also the bulk of the other protein-bound mucopolysaccharides present in the serum decreases in cases with parenchymatous liver disease. The main fractions of the serum polysaccharides differ in their chemical constitution from the glucuronic acid containing polysaccharides found in solid tissues; therefore it is not probable that polysaccharide material released from the tissues is an important direct source of these main fractions of protein-bound serum carbohydrate.

On the other hand, many findings have been reported which indicate that some of the polysaccharides accumulating in pathologic sera may originate from damaged tissues. The fact that parathormone administration produces momentaneous increases of polysaccharidemia supports this theory (111).

#### EFFECT OF HORMONAL SYSTEM

The effect of the hormonal system on the mucopolysaccharide content of the tissues and on the carbohydrate groups of the serum proteins has been investigated by many authors. The affected skin of patients with pretibial myxedema contained increased amounts of acid mucopolysaccharides (146, 147). It has been demonstrated in the guinea pig that orbital myxedema produced by thyroidectomy is accompanied by an increase in the concentration of connective-tissue hexosamine (148). An even greater increase results when thyroidectomized guinea pigs are given thyrotropin. Growth hormone, on the other hand, produces an increase in the amounts of connective tissues and hexosamine with no change in the concentration of hexosamine (149). Either thyroidectomy or hypophysectomy is followed by an increase of protein-bound plasma carbohydrate, which is higher and more sustained than that following a sham operation (86). Elevated levels of serum mucoprotein have been described in cases of severe myxedema (150). The deposition of metachromatic mucopolysaccharides in the comb of the rooster is increased by androgen and decreased by estrogen hormones (151, 152).

Investigations of various authors on the effect of adrenal cortex hormone on

the mucopolysaccharide level differ widely in their results. ACTH administration was followed by an increase of the orosomucoid (seromucoid) level in normal persons; the maximal response was observed after two consecutive daily infusions of 30-40 units (111). On the other hand, ACTH administration was without effect on serum polysaccharide in normal rabbits (153). Sustained mucopolysaccharide elevation after ACTH treatment of acute rheumatic fever despite clinical arrest of the disease (154, 155) may be explained by an independent adrenocortical influence on the mucoprotein level (111). An additional increase in polysaccharide levels occurred after ACTH administration to alloxane diabetic rabbits (153). Administration of the insecticide DDD (2,2-bis parachlorophenyl-1,1-dichlorethane), which gives rise to degenerative changes in the fascicular zone of the adrenal cortex, produces a significant decrease of blood polysaccharide level (156).

#### CAPILLARY PERMEABILITY

The metabolic fate of the protein-bound carbohydrate groups is closely connected with that of the polypeptide part of the protein molecules and it seems that the liver is concerned not only with the production but also with the breakdown of a part of the serum polysaccharides. Blood plasma itself has not the ability to split down the polysaccharide groups of the serum proteins.

Some of the mucoproteins, even when their molecular weight is very high, can pass through the capillary walls very quickly. The rapid biologic effect of the gonadotrophic hormones and their excretion through the kidneys show that the normal capillary wall is permeable for at least some substances of this group. Relatively large amounts of protein-bound carbohydrate pass together with the serum proteins through damaged capillary walls. Pleural exudates in cases of exudative pleurisy contain considerable amounts of protein-bound carbohydrate (157) and the carbohydrate:protein ratio of the exudate proteins shows variations similar to those of the serum proteins (55). Also exudates from cantharidin-provoked blisters showed a similar carbohydrate content (157).

The passage of the serum glucoproteins through damaged capillary walls into tissue fluids has been discussed especially with regard to the carbohydrate-rich hyaline deposits observed in the retina vessels and in the renal glomeruli of some diabetics (158, 159). The amount of protein-bound carbohydrate was higher in the serum of diabetics with these hyaline vascular changes than in the serum of diabetics in whom no sign of hyaline vascular changes were observed (160-162).

#### AMYLOID DEPOSITS

In mice repeatedly injected with casein the development of amyloid changes in the tissues was preceded and accompanied by an increase of the carbohydrate-rich  $\alpha_2$  and  $\beta$  serum globulins (163), indicating that some of the

amyloid deposits may originate from this source. While tissue polysaccharides as hyaluronic acid or chondroitin-sulfuric acid do not give the color reactions of neuraminic acid (164), in amyloidic liver relatively large amounts of methoxyl-neuraminic acid have been found (165). But generally polysaccharide increases in serum and tissues seem to be parallel with metabolic responses caused by the same pathologic process.

#### CEREBROSPINAL FLUID

Protein-bound carbohydrate is also present in the cerebrospinal fluid (Table 3), the total polysaccharide amount in normal cerebrospinal fluid corresponding to about 6.75 mg. per 100 ml. hexoses (166). Although in the proteins of the cerebrospinal fluid the hexose: protein ratio is very similar to that of the serum proteins (166), the hexose:hexosamine ratio is higher in the glucoproteins of the cerebrospinal fluid (167). In patients with an increased carbo-

Table 3. PROTEIN IN CEREBROSPINAL FLUID

<i>Cerebrospinal fluid</i>	<i>No. cases</i>	<i>Protein (mg./100 ml.)</i>	<i>Protein-bound hexoses (mg./100 ml.)</i>	<i>Hexose:protein ratios <math>\times 100</math></i>
Normal	31	326	6.75	2.14
General infectious diseases	12	196	7.41	4.2
Meningitis	19	1000	21.4	2.16
Nephroses	11	150	17.68	6.36

hydrate content of the serum proteins, a similar increase is also found in the proteins of the cerebrospinal fluid. In meningitis the polysaccharide content of the cerebrospinal fluid is raised in a similar degree as its protein content and the carbohydrate:protein ratio does not change significantly. In pathologic conditions, in which the protein concentration of the cerebrospinal fluid is diminished, the glucoprotein content of the cerebrospinal fluid shows a marked increase. In nephrotic patients we found sometimes a sixfold to sevenfold increase of the carbohydrate:protein ratio (168).

#### URINARY MUCOPROTEIN

Normal urine contains relatively large amounts of mucoprotein and the excretion of urinary mucoproteins shows an important elevation in some pathologic conditions. The total amount of excreted urinary mucopolysaccharides can be estimated by determination of the carbohydrate constituents in the dialyzed urine. Dialyzed urine of normal male subjects contained about 29 mg. hexosamine, 38 mg. hexose, 6 mg. fucose, and 6 mg. hexuronic acid pro die (169). Like the polysaccharides present in blood serum, urinary mucopolysaccharides are chiefly prosthetic groups of mucoproteins.

Some of the mucoproteins present in urine have been prepared in a purified

state. Tamm and Horsfall (170) isolated a mucoprotein fraction from normal urine by precipitation with a 0.58M NaCl solution. This mucoprotein has a high molecular weight of the order of  $7.0 \times 10^6$  (171,172), consists of threadlike molecules (173), and inhibits the hemagglutination by influenza virus and some other viruses (171, 174).

The chemical constitution and biologic properties of this mucoprotein have been investigated simultaneously by Gottschalk (175) and Odin (176) and the presence of 8.4–9.0 per cent hexose, 1.1 per cent fucose, 7–9 per cent hexosamine, 9.1 per cent neuraminic acid, and small amounts of sulfate S has been established. Anderson and MacLagan (177) obtained a mucoprotein fraction of a similar composition from urine by the use of adsorption on benzoic acid. When subjected to paper electrophoresis this fraction was found to migrate as a diffuse band with a mean mobility between that of serum  $\alpha_1$ - and  $\alpha_2$ -globulin. In urinary mucoprotein excretion a similar sex difference has been shown as in the mucoprotein content of the blood serum: 24-hour urine contained an average of 146 mg. of this mucoprotein in man and 106 mg. in women (177). No significant diurnal rhythm could be observed in the excretion of this mucoprotein fraction.

Boyce and Swanson (178) ultrafiltrated urine of normal adults and of patients with renal calculus disease and extracted the residue with a molar NaCl solution. The NaCl-insoluble fraction of the residue was shown to be an electrophoretically homogeneous mucoprotein fraction containing almost exactly the same amounts of hexosamine, hexoses, fucose, and sialic acid as the mucoprotein fraction prepared by Tamm and Horsfall. Normal subjects excreted an average of 44 mg. of this mucoprotein fraction per day. This amount corresponds to about 30 per cent of the mucoprotein fraction obtained by adsorption on benzoic acid and to less than 10 per cent of the total mucoprotein content of the normal urine as determined by the analysis of nonfractionated dialysed urine.

In the urine of patients with cutaneous ureterostomy only very small amounts (12–18 mg. per day) of this mucoprotein could be detected (178), and it is therefore probable that the bulk of this urinary mucoprotein fraction is secreted by the epithelium of the lower parts of the urinary tract (179).

Another fraction of urinary mucopolysaccharides seems to be responsible for a very interesting reaction given by some urines and known as the obstacle of Donaggio (180–183). While in normal urine a violet precipitate is formed after addition of thionin and ammonium molybdate, in urine excreted during states of strong muscular fatigue the formation of this precipitate is inhibited. The same inhibition is seen in typhoid fever and malignant disease (186), and in salmonellosis and in normal urines of carnivorous animals a positive Donaggio reaction has been observed. The substances causing this inhibition are two mucoprotein fractions with an electrophoretic velocity corresponding to the  $\alpha_1$ - and  $\gamma$ -globulins respectively. A Donaggio-active mucoprotein fraction isolated

by zone electrophoresis had a composition different from that of the other mucoprotein fractions hitherto isolated from urine. The polypeptide component contains more phenylalanine and less hydroxyproline than the mucoprotein prepared by adsorption on benzoic acid and the hexosamine:hexose ratio of the carbohydrate component is higher than that of the virus-inhibiting mucoprotein analyzed by Gottschalk and Odin (187).

Urinary mucoprotein secretion has a close relation to the pathogenesis of renal calculus disease. Some mucoprotein fractions seem to facilitate the formation of urinary concretions and the amount of the mucoprotein fraction prepared by the method of Boyce and Swanson shows a threefold increase in patients with renal calculus disease (178). Other fractions of urinary mucopolysaccharides seem to have a stabilizing effect on urines oversaturated with crystalloids.

Beside the hexose-containing mucopolysaccharide fractions referred to above, glucuronic-acid-containing mucopolysaccharides are also present in normal human urine. The 24-hour excretion of acid mucopolysaccharides (expressed as bound glucuronic acid) was 4.9 mg. in males and 3 mg. in females (188). These acid mucopolysaccharides were found also in urine obtained from the renal pelvis, hence did not originate from secretions of the lower urinary tract. Prostatic massage did not increase the amount of this material. The main fraction of this acid mucopolysaccharide showed the same  $R_f$  value as chondroitin sulfate, and produced a single spot when mixed with chondroitin sulfate in a single solvent system. Hyaluronic acid and mucoitin-sulfuric acid injected in rabbits intravenously are excreted in the urine and also after injections of heparin metachromatic degradation products could be detected in the urine (190). Subcutaneous injection (but not direct addition to urine) of hyaluronidase increased the protective colloidal activity of urine, suggesting that this enzyme acts indirectly by releasing hyaluronate from the site of injection, which is subsequently excreted with urine (189, 191).

### CONCLUSION

The tremendous research work done in these last years by a great number of laboratories has shown that the mucoproteins and mucopolysaccharides form an important part of the complex colloid systems present in the blood serum, tissue fluids, secretions, and excretions and that these substances are in a very close relation to the response of the living cells to pathogenic factors.

### REFERENCES

1. Werner, I., *Acta Physiol. Scand.* **19**, 27 (1949).
2. Stary, Z., Bursa, F., and Anhegger-Lisie, S. G., *Zeitschr. f. Physiol. Chem.* **295**, 29 (1953).
3. Böhm, P., and Baumeister, S. L., *Kli. Wo.* **33**, 712 (1955).
4. Stary, Z., and Cindi, R., *Bull. Fac. Méd. Istanbul* **18**, 69 (1955).
5. Stary, Z., and Cindi, R., *Naturwissenschaften* **43**, 179 (1956).

6. Mayer, K., *Adv. Protein Chemistry* **2**, 250 (1945).
7. Klenk, E., *Angew. Chemie* **68**, 349 (1956).
8. Klenk, E., *Zeitschr. Physiol. Chem.* **268**, 50 (1941).
9. Klenk, E., and Lauenstein, K., *Zeitschr. Physiol. Chem.* **291**, 147 (1952).
10. Klenk, E., and Faillard, H., *Zeitschr. Physiol. Chem.* **298**, 230 (1954).
11. Klenk, E., Faillard, H., and Lempfried, H., *Zeitschr. Physiol. Chem.* **301**, 235 (1955).
12. Blix, G., *Zeitschr. Physiol. Chem.* **240**, 43 (1936).
13. Blix, G., Swennerholm, L., and Werner, I., *Acta Med. Scand.* **6**, 358 (1952).
14. Blix, G., Lindberg, E., Odin, L., and Werner, I., *Nature (London)* **175**, 340 (1955).
15. Werner, I., and Odin, L., *Acta Soc. Med. Upp.* **57**, 230 (1952).
16. Kuhn, R., Brossmer, R., and Schulz, W., *Chem. Ber.* **87**, 123 (1954).
17. Kuhn, R., and Brossmer, R., *Angew. Chem.* **68**, 211 (1956).
18. Yamakawa, T., and Suzuki, S., *J. Biochem.* **42**, 727 (1955).
19. Yamakawa, T., and Suzuki, S., *J. Biochem.* **38**, 199 (1951).
20. Yamakawa, T., and Suzuki, S., *J. Biochem.* **39**, 175 (1952).
21. Mayer, K., *Advances in Protein Chemistry* **2**, 250 (1945).
22. Gottschalk, A., *Yale J. Biol. Med.* **28**, 525 (1956).
23. Zilliken, F., Braun, G. A., and György, P., *Arch. Biochem. Biophys.* **54**, 564 (1955).
24. Hoover, R. E., Braun, G. A., and György, P., *Arch. Biochem. Biophys.* **47**, 216 (1953).
25. Gottschalk, A., and Lind, R. E., *Nature (London)* **164**, 232 (1949).
26. Gottschalk, A., *Nature (London)* **167**, 845 (1951).
27. Boas, N. F., and Foley, J. B., *Proc. Soc. Exp. Biol. Med.* **86**, 690 (1954).
28. Altschuler, C. H., and Angevine, D. M., *Amer. J. Pathol.* **25**, 1061 (1949).
29. Catehpole, H. R., *Proc. Soc. Exp. Biol. Med.* **75**, 221 (1950).
30. Zanetti, C. U., *Gazz. chim. ital.* **33** I, 160 (1903).
31. Werner, I., and Odin, L., *Experientia* **5**, 233 (1949).
32. Sörensen, M., and Haugaard, G., *Biochem. Z.* **260**, 247 (1933).
33. Rimington, C., *Biochem. J.* **23**, 430 (1929); **25**, 1061 (1931).
34. Böhm, P., and Baumeister, L., *Kli. Wo.* **32**, 611 (1954); *Zeitschr. Physiol. Chem.* **300**, 153 (1955).
35. Micheel, F., and Suthaus, F., *Naturwissenschaften* **43**, 108 (1956).
36. Werner, I., *Acta Chem. Scand.* **5**, 1396 (1951).
37. Odin, L., and Werner, I., *Acta Soc. Med. Uppsal.* **57**, 227 (1952).
38. Waldron, D. M., *Nature (London)* **170**, 461 (1952).
39. Berkman, J., Rilkin, H., and Ross, G., *J. Clin. Invest.* **32**, 415 (1953).
40. Stary, Z., and Yuvanidis, M., *Biochem. Zeitschr.* **324**, 206 (1953).
41. Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R., *Proc. Soc. Exp. Biol. Med.* **69**, 507 (1948).
42. Stary, Z., Bursa, F., Kaleoglu, Ö., and Bilen, M., *Bull. Fac. Méd. Istanbul* **13**, 233 (1950).
43. Böhm, P., St. Dauber, and Baumeister, L., *Kli. Wo.* **32**, 289 (1954).
44. Shetlar, M. R., and Shetlar, C. L., *Proc. Soc. Exp. Biol. Med.* **88**, 622 (1955).
45. Boas, N. F., and Peterman, A. F., *Proc. Soc. Exp. Biol. Med.* **82**, 19 (1953).
46. Stary, Z., Bursa, F., Kaleoglu, Ö., and Bilen, M., *Bull. Fac. Méd. Istanbul* **13**, 259 (1950).
47. Stary, Z., Bursa, F., Kaleoglu, Ö., and Bilen, M., *Bull. Fac. Méd. Istanbul* **13**, 453, (1950).
48. Pedersen, K. O., *J. Physic. Colloid Chem.* **51**, 164 (1947).
49. Klenk, E., and Stoffel, J., *Zeitschr. Physiol. Chem.* **302**, 286 (1955).
50. Boas, N. F., and Peterman, A. F., *Proc. Soc. Exp. Biol. Med.* **82**, 19 (1953).
51. Werner, I., *Acta Physiol. Scand.* **19**, 27 (1949).

52. Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.* **26**, 90 (1947).
- 52a. Seibert, F. B., Pfaff, M. L., and Seibert, M. V., *Arch. Biochem. Biophys.* **18**, 279 (1949).
53. Seibert, F. B., and Seibert, M. V., *Amer. Rev. Tuberculosis* **62**, 67 (1950).
54. Stary, Z., Bodur, H., Lisie, S. G., and Batiyok, F., *Kli. Wo.* **1953**, 399.
55. Stary, Z., Akkurt, S., and Bodur, H., *Med. Monatsschrift* **7**, 772 (1954).
- 55a. Galetti, F. G., Gelli, G., and Loli-Piccolomini, M., *Archivio die Patologia* **31**, 307 (1955).
56. Stary, Z., Bursa, F., Kaleoglu, Ö., and Bilen, M., *Med. Monatsschrift* **6**, 497 (1953).
57. West, R., Clarke, D. H., and Kennedy, E. M., *J. Clin. Invest.* **17**, 173 (1938).
58. Nilsson, I., *Biochem. Z.* **291**, 254 (1937).
59. Hammerström, R. N., Adams, F. H., Bussman, J., and Lillihei, C. W., *Proc. Soc. Exp. Biol. Med.* **79**, 527 (1952).
60. Lustig, B., and Langer, A., *Biochem. Z.* **242**, 321 (1931).
61. Clemens, H. J., *Acta Histochemica* **2**, 170 (1956).
62. Gamp, A., *Zeitschr. f. Rheumaforschg.* **14**, 167 (1955).
63. Rosenberg, C., and Schloss, B., *Amer. Heart J.* **38**, 872 (1949).
64. Shetlar, M. R., Payne, R. W., Bullock, J. A., Patrick, D. R., Hellbaum, A. A., and Ishmall, W. K., *J. Clin. Invest.* **32**, 1208 (1953).
65. Payne, R. W., Shetlar, M. R., Bullock, J. A., Patrick, D. R., Hellbaum, A. A., and Ishmall, W. K., *Amer. Int. Med.* **41**, 775 (1954).
66. Boas, N. Y., Bollet, A. J., and Bunim, J. J., *J. Clin. Invest.* **34**, 782 (1955).
67. Stary, Z., Kaleoglu, Ö., and Bursa, F., *Istanbul Contribution to Clinical Science* **1**, 311 (1951).
68. Lustig, B., and Nassau, E., *Exp. Medicine and Surgery* **4**, 255 (1946).
69. Novak, J., and Lustig, B., *J. Mount Sinai Hosp.* **14**, 534 (1947).
70. Shetlar, M. R., Kelly, K. H., Foster, J. V., Shetlar, C. L., and Everett, M. R., *Amer. J. Obst. Gynecol.* **59**, 1140 (1950).
71. Stary, Z., Bursa, F., Tezok, O., and Cindi, R., *Zeitschr. f. Physiol. Chem.* **228**, 55 (1951).
- 71a. Farina, L., Galletti, F., Giungi, F., and Loli-Piccolomini, M., *Rivista ital. Ginecologia* **37**, 257 (1955).
72. Stary, Z., Erez, N., Bodur, H., Anhegger-Lisie, S. G., and Cepeloglu, R., *Arch. Gynaekol.* **184**, 330 (1954).
73. Stary, Z., Kaleoglu, Ö., and Bursa, F., *Istanbul Contrib. to Clin. Science* **1**, 158 (1951).
74. Shetlar, M. R., Shetlar, C. L., Richmond, V., and Everett, M. R., *Cancer Res.* **10**, 681 (1950).
75. Shetlar, M. R., Bullock, J. A., Shetlar, C. L., and Payne, R. W., *Proc. Soc. Exp. Biol. Med.* **88**, 107 (1955).
76. Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.* **27**, 617 (1949).
77. Merten, R., *Biochem. Z.* **297**, 304 (1938).
78. Shetlar, M. R., *Texas Rep. Biol. Med.* **10**, 228 (1952).
79. Catchpole, H. R., *Proc. Soc. Exp. Biol. Med.* **75**, 221 (1950).
80. Nisselbaum, J. S., and Bernfield, P., *J. Amer. Chem. Soc.* **78**, 687 (1956).
81. Shetlar, M. R., Erwin, C. D., and Everett, M. R., *Cancer Res.* **10**, 445 (1950).
82. Roboz, S., Hess, W. C., and Foster, F. M., *Arch. Neur. Psych.* **73**, 536 (1955).
83. Knobloch, W. H., Nagle, P., Shetlar, C. L., and Shetlar, M. R., *Proc. Soc. Exp. Biol. Med.* **81**, 417 (1952).
84. Keyser, J. W., *J. Clin. Pathol.* **5**, 194 (1952).



85. Shetlar, M. R., Knobloch, W. H., Richmond, V., Shetlar, C. L., and Everett, M. R., *Proc. Soc. Exp. Biol. Med.* **83**, 75 (1953).
86. Boas, N. F., and Foley, J. B., *Endocrinology* **56**, 305 (1955).
87. Shetlar, M. R., Bryan, R. S., Forster, J. V., Shetlar, C. L., and Everett, M. R., *Proc. Soc. Exp. Biol. Med.* **72**, 294 (1949).
88. Pirano, C. L., and Catchpole, H. R., *Arch. Pathol.* **51**, 597 (1951).
89. Küley, M., Bodur, H., and Stary, Z., First International Congress for Allergy 1951, p. 363.
90. Arnaki, M., (Istanbul) Unpublished data.
91. Blix, G., Tiselius, A., and Svensson, H., *J. Biol. Chem.* **137**, 485 (1941).
92. Boas, N. F., *Proc. Soc. Exp. Biol. Med.* **90**, 4 (1955).
93. Köiw, E., and Grönwall, A., *Scand. J. Clin. Lab. Invest.* **4**, 244 (1952).
94. Stary, Z., and Ugur, A., *Kli. Wo.* 766 (1955).
95. Björnesjö, K. B., *Scand. J. Clin. Lab. Invest.* **7**, 153 (1955).  
Uzman, L.
96. Laurell, C. B., and Skoog, N., *Scand. J. Clin. Labor. Invest.* **8**, 21 (1956).
97. Wunderly, C., and Piller, S., *Kli. Wochschr.* **32**, 425 (1954).
98. Weimer, H. E., Moshin, J. R., and Nelson, E. L., *J. Immol.* **74**, 243 (1955).
99. Weimer, H. E., Jameson, E., Moshin, J. R., Quinn, F. A., and Nelson, E. L., *Proc. Soc. Exp. Biol. Med.* **38**, 571 (1953).
100. Arnaki, M., Soysal, S. S., and Stary, Z., unpublished data.
101. Weimer, H. E., Mehl, J. W., and Winzler, R. J., *J. Biol. Chem.* **185**, 561 (1950).
102. Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.* **27**, 609 (1948).
103. Smith, E. M., Brown, D. M., Weimer, H. E., and Winzler, R. J., *J. Biol. Chem.* **185**, 569 (1950).
104. Schmid, K., *J. Amer. Chem. Soc.* **72**, 2816 (1950).
105. Schmid, K., *J. Amer. Chem. Soc.* **75**, 60 (1953), *Biochim. Biophys. Acta* **14**, 437 (1954).
106. Weimer, H. E., Vogl, M., Quinn, F. A., and Redlich-Moshin, J., *Proc. Soc. Exp. Biol. Med.* **90**, 494 (1955).
107. Weimer, H. E., and Winzler, R. J., *Proc. Soc. Exp. Biol. Med.* **90**, 458 (1955).
108. Greenspan, E. M., *Adv. Internal Med.* **7**, 101 (1955).
109. De La Huerga, J., Dubin, A., Kushner, D. S., Dyniewicz, H. A., and Popper, H., *J. Lab. Clin. Med.* **47**, 403 (1956).
110. Greenspan, E. M., and Drabing, D. A., *Arch. Intern. Med.* **91**, 474 (1953).
111. Kushner, D. S., Honig, K., Dubin, A., Dyniewicz, H. A., Bronsky, D., De la Huerga, J., and Popper, H., *J. Lab. Clin. Med.* **47**, 409 (1956).
112. Seibert, F. B., Seibert, M. V., Aino, A. J., and Campbell, H. W., *J. Clin. Invest.* **26**, 90 (1947).
113. Weimer, H. E., Bock, R. A., Carpenter, C. M., Redlich-Moshin, J., Drusch, H. E., and Miller, J. N., *J. Inf. Dis.* **96**, 19 (1955).
114. Criscione, C., and Mariano, R., *Aggiornamento paediatrico* **6**, 1 (1955).
115. Henry, R. J., Berkman, S., Little, M. S., and Winzler, R. J., *J. Amer. Med. Assoc.* **147**, 37 (1951).
116. Greenspan, E. M., Lehman, I., Graff, M. M., and Schoenbach, E. B., *Cancer* **4**, 972 (1951).
117. Greenspan, E. M., Tepper, B., Terry, L. L., and Schoenbach, E. B., *J. Lab. Clin. Med.* **39**, 44 (1952).
118. Mandel, E. E., Gorsuch, T. L., and Jones, F. L., *Amer. J. Med.* **16**, 905 (1954).
119. Stary, Z., *8. Intern. Congr. Rheum. Diseases*, Geneva, 1953, Summ. of Comm. p. 127.
120. Bergsterman, H., *Kli. Wochschr.* **17**, 392 (1952).



121. Stary, Z., Bodur, H., and Batiyok, F., *Schweis. Med. Wochenschr.* **81**, 1273 (1951).
122. Cohen, R. J., and Byham, B., *J. Lab. Clin. Med.* **35**, 869 (1950).
123. Greenspan, E. M., *Science* **114**, 395 (1951).
124. Allen, J. G., and Jacobson, L., *Science* **105**, 388 (1947).
125. Abernethy, T. J., and Avery, O. T., *J. Exp. Med.* **73**, 173 (1941).
- 125a. McLeod, C. M., and Avery, O. T., *J. Exp. Med.* **73**, 183, 191 (1941).
126. Jacques, L. B., Monkhouse, F. C., and Stewart, M., *J. Physiol. (London)* **109**, 41 (1949).
127. Glick, D., and Sylven, B., *Science* **113**, 388 (1951).
128. Glick, D., Good, R. A., Kelley, V. C., Winzler, R. J., and Mehl, J. W., *Proc. Soc. Exp. Biol. Med.* **71**, 412 (1949).
129. Wattenberg, L. W., and Glick, D., *Arch. Biochem. Biophys.* **35**, 290 (1952).
130. Glick, D., and Moore, D. H., *Arch. Biochem.* **19**, 173 (1948).
131. Moore, D. H., and Harris, N., *J. Biol. Chem.* **179**, 377 (1949).
132. Glick, D., and Gollan, F., *J. Infect. Dis.* **83**, 200 (1948).
133. Grais, M., and Glick, D., *J. Infect. Dis.* **85**, 101 (1949).
134. Glick, D., and Campbell, B., *Proc. Soc. Exp. Biol. Med.* **70**, 29 (1949).
135. Thompson, R. T., and Moses, F. E., *Fed. Proc.* **7**, 282 (1948).
136. Good, R. A., and Glick, D., *J. Infect. Dis.* **86**, 38 (1950).
137. Kelley, V. C., Good, R. A., and Glick, D., *J. Clin. Invest.* **29**, 1500 (1950).
138. Hakanson, E. Y., and Glick, D., *J. Nat. Cancer Inst.* **9**, 129 (1948).
139. Grais, M., and Glick, D., *J. Invest. Dermatol.* **11**, 259 (1948).
140. Good, R. A., Kelley, V. C., Good, T. A., and Glick, D., *Pediatrics* **12**, 575 (1953).
141. Hakanson, E. Y., and Glick, D., *J. Clin. Invest.* **28**, 713 (1949).
142. Snively, G. G., and Glick, D., *J. Clin. Invest.* **29**, 1087 (1950).
143. Good, T. A., Good, R. A., Kelley, V. C., and Glick, D., *Amer. J. Physiol.* **166**, 555 (1951).
144. Kiriluk, L. B., Kremen, A. J., *J. Natl. Cancer Inst.* **11**, 993 (1950).
145. Greenspan, E. M., *Adv. Internal. Med.* **7**, 101 (1955).
146. Watson, E. M., and Pearce, R. H., *Amer. J. Clin. Pathol.* **17**, 507 (1947).
147. Watson, E. M., and Pearce, R. H., *Amer. Rev.* **19**, 442 (1949).
148. Ludwig, A. W., Boas, N. F., and Soffer, L. J., *Proc. Soc. Exp. Biol. Med.* **73**, 137 (1950).
149. Boas, N. F., and Foley, J. B., *Proc. Soc. Exp. Biol. Med.* **87**, 89 (1954).
150. Mancini, E. E., Garberi, J. C., and de la Balze, F. A., *Rev. Soc. Argent. de Biol.* **27**, 285 (1951).
151. Boas, N. F., and Ludwig, A. W., *Endocrinology* **46**, 299 (1950).
152. Ludwig, A. W., and Boas, N. F., *Endocrinology* **46**, 291 (1950).
153. Törnblom, N., *Acta Med. Scand.* **153**, 143 (1955).
154. Adams, F. H., Kelly, V. C., Dwan, P. F., and Glick, D., *Pediatrics* **7**, 472 (1951).
155. Kelly, V. C., Adams, F. H., and Good, R. A., *Pediatrics*, **12**, 607 (1953).
156. Törnblom, N., *Acta Med. Scand.* **53**, 149 (1955).
157. Lustig, B., and Nassau, E., *Amer. Rev. of Tuberculosis* **43**, 817 (1941).
158. Frank, E., *Istanbul Contrib. Clin. Science* **2**, 157 (1952).
159. Alatas, S., *1st Contrib. Clin. Science* **3**, 164 (1955).
160. Nielsen, G. H., and Poulsen, J. E., *Reports Steno Memorial Hosp. (Copenhagen)* **5**, 71 (1953).
161. Poulsen, J. E., *Reports Steno Memorial Hosp. (Copenhagen)* **5**, 94 (1953).
162. Törnblom, N., and Nordström, K., *Acta endocrinol.* **17**, 426 (1954).
163. Ott, H., and Schneider, G., *Zeitschr. f. Ges. Exp. Med.* **116**, 545 (1951).
164. Gottschalk, A., *Yale J. Biol. Med.* **28**, 525 (1956).

165. Klenk, E., and Faillard, H., *Zeitschr. Physiol. Chem.* **229**, 191 (1955).  
166. Stary, Z., Bodur, H., and Lisie, S. G., *Kli. Wochschr.* **339**, 1953.  
167. Roboz, E., Murphy, J. B., Hess, W. C., and Forster, F. M., *Proc. Soc. Exp. Biol. Med.* **89**, 691 (1955).  
168. Stary, Z., Soysal, S. S., and Anhegger, S. L., *Kli. Wochschr.* (in press).  
169. Hamerman, D., and Hatch, F. T., *Proc. Soc. Exp. Biol. Med.* **89**, 279 (1955).  
170. Tamm, I., and Horsfall, F. I., *Proc. Soc. Exp. Biol. Med.* **71**, 108 (1950).  
170a. Tamm, I., Bugher, J. C., and Horsfall, F. L., *J. Biol. Chem.* **212**, 125 (1955).  
171. Tamm, I., and Horsfall, F. I., *J. Exp. Med.* **95**, 71 (1952).  
172. Perlmann, G. E., Tamm, I., and Horsfall, F. L., *J. Exp. Med.* **95**, 99 (1952).  
173. Porter, K. R., and Tamm, I., *J. Biol. Chem.* **212**, 135 (1954).  
174. Hirst, G. K., *J. Exp. Med.* **76**, 195 (1942), **78**, 99 (1943).  
175. Gottschalk, A., *Nature (London)* **170**, 662 (1952).  
176. Odin, L., *Nature (London)* **170**, 662 (1952).  
177. Anderson, A. J., and MacLagan, N. F., *Biochem. J.* **59**, 638 (1955).  
178. Boyce, W. H., and Swanson, M., *J. Clin. Invest.* **34**, 1581 (1955).  
179. Boyce, W. H., Garvey, F. K., and Norfleet, C. M., *J. Urol.* **72**, 1019 (1952).  
180. Tayeau, F., Biserte, G., Montreuil, J., and Marqueville, S., *Compt. Rend. Acad. Sci.* **237**, 208 (1953).  
181. Tayeau, F., *Exposé Annuel Biochim. Méd.* **16**, 215 (1954).  
182. Tayeau, F., *Tunisie Méd.* **39**, 664 (1951).  
183. Biserte, G., Tayeau, F., Montreuil, J., Holleman, J., and Dautrevaux, M., *Clin. Chim. Acta (Amsterdam)* **1**, 115 (1956).  
184. Waldenström, J., *Schw. Med. Wochenschr.* **927**, 1948.  
185. Pernis, B., Wuhrmann, F., and Wunderly, C., *Acta Haematol.* **11**, 309 (1954).  
186. Donaggio, A., *Boll. Soc. ital. Biol. Sperim.* **8**, 1436 (1933).  
187. Biserte, G., Tayeau, F., Montreuil, J., Hollman, J., and Dautrevaux, M., *Clin. Chim. Acta (Amsterdam)* **1**, 115 (1956).  
188. Kerby, G. P., *J. Clin. Invest.* **33**, 1168 (1954).  
189. Butt, A. J., Hauser, E. A., and Seifter, J., *J. Amer. Med. Assoc.* **150**, 1096 (1951).  
190. Jaques, L. B., Napke, E., and Levy, S. W., *Circulation Research* **1**, 321 (1953).  
191. Butt, A. J., *J. of Urology* **67**, 450 (1952).  
192. Jimenez Diaz, C., Aguirre, M., and Arjona, E., *Bull. Inst. Med. Research, Madrid* **6**, 137 (1953).  
193. Koyser, J. W., *J. Clin. Pathol.* **5**, 194 (1952).  
193a. Piccini, L., and Villani, E., *La Clinica* **16**, 2 (1956).

# Significance of Lipoproteins in Clinical Chemistry

J. C. M. Verschure

URGED ON BY THE DRAMATIC frequency of myocardial infarction, a feverish research activity has developed in the field of the chemistry of the lipoproteins. The clinician asks in the first place for a test that would enable him to decide if and to which degree atheromatosis is present. The traditional line of thinking, implying that cholesterol and lipid metabolism are related to this condition has received numerous new impulses. The quickly growing amount of information is not yet suitable for a schematic treatment. Most of the problems are still unsolved and a clear concept has not yet been obtained. This review tries to summarize and to show the red threads that mark the major lines of development. At this time, it is impossible to mention every author or attempt a proper distribution of credit.

It was only in the forties that fundamental research work on lipoproteins was started. Reviews are available on the earlier history and chemistry of the lipoproteins and data may be found there (22, 10). The main objective of the past few years has been the separation of the various lipoproteins in serum. The remarkable achievements of Gofman and his group focused general attention upon them and their relationship with atheromatosis. However, simpler methods were necessary to enable the hospital laboratories to start work on the lipoproteins. Fortunately various new approaches have been opened up in the last 5 years and some proved most fruitful for clinical chemistry. Better techniques of ethanol or salt precipitation fractionation methods opened simpler ways of separation, based upon the various solubilities of the fractions. However, these techniques were quickly overgrown in the hospital laboratories by the attractive possibilities of paper electrophoresis combined with a lipid-staining method.

It is a remarkable fact that these three approaches are derived from protein chemistry. The separations thus depend largely upon the protein moieties of the particles and consequently less information is obtained about the lipids. These were studied up till very recently practically only with the classic esti-

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mations for cholesterol, phospholipids, and neutral fats. Newer technics from the field of lipid chemistry are rarely used, but must be expected to become indispensable for the solution of the underlying problems.

Dynamic studies found a mighty stimulus through the discovery of the "clearing factor."

From this rich field of possibilities I choose for this review those methods that may be applicable in the clinical chemical laboratory both for routine and for research work; namely:

1. Critical review on the possibilities of lipoprotein separation by means of paper electrophoresis
2. Other methods of separation
3. Dynamic studies by lipoprotein lipase systems
4. Lipoprotein flocculation tests
5. Clinical aspects of chemical data

### LIPID DIAGRAMS WITH PAPER ELECTROPHORESIS

If one takes a series of lipid diagrams as might be made in every hospital laboratory, using a quick method of electrophoresis (126) and the Sudan black stain, numerous interesting phenomena may be seen, e.g., the separation into two groups of lipoproteins, alpha and beta, the trail in the region of the gamma globulins, abnormal mobility of the beta group in several cases of lipid nephrosis and other conditions, remarkably intense "trail" in cases of "essential hyperlipimia," very distinct and intensive beta band in cases of xanthomatosis, abnormal lipid-bearing gamma globulin in cases of gamma myeloma, disappearance of the alpha band in cases of biliary obstruction, etc. Such qualitative findings are described by many authors (122, 123, 109, 2, 35) and they are not subject to much controversy. In some instances the results are superior to combined analysis with fluid electrophoresis and ultracentrifugal experiments (109, 74).

### TECHNICAL DIFFICULTIES IN QUANTITATIVE EVALUATION

As soon as we try to use the results described for quantitative evaluation, numerous difficulties are encountered. Many factors that influence the results have been studied already:

#### TIME OF COLLECTING THE BLOOD SAMPLE

The total cholesterol and phospholipid contents of the blood were thought to be practically independent of meals, sleep, or the time at which the blood sample was taken (12, 75). The content of neutral fats shows large variations and if "total lipids" are to be measured by staining methods on paper strips, blood samples of fasting individuals should be used. In one and the same person, cholesterol and phospholipids have always been supposed to be relatively constant, but over a longer period cholesterol fluctuations of 31 per cent and

phospholipid fluctuations of 23 per cent have been described (134). With careful analysis, the variations in cholesterol level of individuals on a constant regimen, are found to be of more importance than supposed formerly (3). Cyclic variations occur especially in women, related in some way to the menstrual cycle (82). Gaining weight or reducing may have an influence (82) as well as eating habits (77). Thus it becomes increasingly difficult to speak about a "normal value." This is reflected in the high variations of normal values, found by various investigators. Analytic errors and differences in methods complicate the picture (134). Greatest care in analysis and standardization may improve this.

#### STABILITY OF LIPOPROTEINS

For ultracentrifugal separations, serum may be kept in the refrigerator at about 5° C. for several weeks (90). Storage in the frozen state for as long as 6 months did not affect the qualitative results (2). For quantitative analyses freezing seemed to us less satisfactory, especially for pathologic sera with high beta lipoprotein contents as may be obtained from nephrosis or essential hyperlipemia. The lipoproteins are less stable than other serum proteins. Denaturation of lipoproteins increases their adsorption to the cellulose, thus producing faulty results (136). Analysis of fresh serum seems to be preferable. If serum is kept in the incubator at 37° C. the relative concentration of the beta lipoproteins diminishes and that of the alpha lipoproteins increases. This shift proceeds with time.

After completion of the separation on the filter paper strips, these should be analyzed as soon as possible. Langan *et al.* (66) found that after a 4 days' exposure to air at room temperatures, 55 per cent of the cholesterol was lost, apparently by oxidation, and 16 per cent of the nitrogen. However, others (3) are optimistic and found that cholesterol on air-dried strips could be stored for several months at any rate, at a temperature of 25-30° C. Perhaps the presence of other serum proteins in the whole serum has a strongly protective action. This point shows how many contradictions already occur in such basic and easily controllable facts. It shows clearly that the whole field is in its first stages of development and that much critical work is still to be done.

#### ADSORPTION OF LIPOPROTEINS TO THE CELLULOSE

Swahn (122) found that alpha lipoproteins were less bound to the cellulose fiber than beta lipoproteins. Langan (66) further studied the trailing effect. They found a linear recovery of cholesterol in the alpha fraction with increasing amounts of serum. This rules out the possibility of significant adsorption of the alpha fraction, of the kind commonly observed, in which a fairly constant amount of material is adsorbed on a given area of paper, regardless of the amount applied. They believe that albumin, passing over the paper in front of the alpha lipoproteins, covers up the available adsorptive sites on the

paper, thus "laying the carpet" for the globulin fractions. If fresh serum is used, the influence of adsorption must not be overestimated. However, top fractions, obtained with Gofman's technic could not well be separated by paper electrophoresis (114) and adsorption may be one of the causes.

Moreover, chylomicrons remain close to the base line and are difficult to separate from the beta lipoproteins. They are often measured together with them, this procedure having obvious disadvantages.

#### VISUALIZATION OF THE LIPIDS ON THE PAPER STRIPS

Numerous procedures have been proposed for the visualization, using various stains, partly modifications of histologic technics. Oil Red O (31, 64), Sudan Black B (122, 123, 79), Sudan III (34), Sudan IV (103), Cibablau (135), Fettrot 7 B (92), Osmic acid (76), Scharlachrot, iodine, and bromine (127) were used and various modifications were described with these "dyes." Excellent critical studies have been made on the Sudan Black stain by Swahn (123) and on the Oil Red O stain by Jencks and Durrum (55). We will restrict our discussion to these two methods.

Most workers agree to the fact that, in general, staining of the lipids is a consequence of the physical distribution of dye between the lipid and the staining solution, and that the staining does not represent a chemical binding between dye and lipid. The more favorable this distribution coefficient and the less dye adsorbed to the filter paper, the more contrast the diagrams will show. If the staining solution contains too high a percentage of organic solvents, the distribution coefficient will be influenced unfavorably and, moreover, an increasing amount of the lipids will be washed out. Jencks and Durrum found that with a 60 per cent ethanol-water solution without dye, 25 to 30 per cent of the serum lipids was dissolved. If the percentage of organic solvents is taken too low, precipitation of dye on proteins and on cellulose fibers causes difficulties. Many methods are therefore based upon solvent concentrations that are empirically chosen. The "staining" process is still more complicated by the fact that both the lipids to be stained, and the dye in the staining solution are mixtures of various components. Both Sudan Black B (80) and Oil Red O (55) appeared to contain at least four substances. In the staining process these components influence each other in a hitherto unknown way. Different lots of dye proved to stain differently. The absolute uptake of dye varied up to twofold in the case of Oil Red O. The lipids contain both free and esterified cholesterol, phospholipids, mainly lecithins and cephalins, and various neutral fats. Concerning the uptake of dye by these substances, no agreement exists. Swahn (124) found that triolein, lecithin, corn oil, and serum lipids show the same uptake of dye. This is also true for tristearin, cholesterol, and cholesterylstearate in the presence of serum lipids. Jencks and Durrum (55) found that isolated cholesterol and various fatty acids do not take up appreciable amounts of Oil Red O or Sudan Black B. The dye uptake of

cholesterol was not significantly increased by the addition of triolein. The hypothesis that only those lipids that are in the liquid state take up dyes, led to contradictory results in their work. It seems certain that the serum phospholipids stain readily.

#### QUANTITATIVE EVALUATION OF STAINED DIAGRAMS

From these short notes it is evident that the quantitative estimation in lipid diagrams is subject to a great number of systematic and accidental errors. The systematic errors must be ruled out as much as possible by a very careful choice of the experimental conditions. It is dangerous to use insufficiently studied methods for quantitative estimations although "beautiful" diagrams are obtained, which may be sufficient for qualitative purposes. More basic work has to be done in this field. The systematic errors that cannot be avoided, should be known, e.g., as the lack of linearity between beta lipoprotein concentration and Oil Red O uptake in cases of a high beta lipid level (55). The accidental errors must be avoided by painstaking accuracy and strict standardization for the filter paper as well as for dye, concentration of the staining solution, temperature of staining, time, rinsing, etc. This allows at least quantitative comparative work in the same laboratory. Under such conditions reproducibility was found satisfactory by several authors (123, 55, 87). Direct comparison of the results of various laboratories is disappointing. Even with the less complicated methods of serum protein estimation by means of paper electrophoresis, this is still impossible (33), even though we know many more basic facts about these methods.

#### USE OF A STANDARD

The introduction of a standard seems promising at first sight. However, the use of a known quantity of some pure stainable lipid or mixture of pure lipids has the disadvantage that it cannot be compared with the serum lipids and their behavior toward the dye. To use it as a direct measure for the quantity of lipids present in the serum fractions is certainly not permitted. Moreover, the dye uptake of such spots showed in our laboratory a much greater variation than that of triple measurements in serum fractions. However, Wunderly gets good results with an artificial standard (137). The use of serum spots as standards introduces other difficulties. The spots show other staining properties than the lipid bands in the diagrams. From such spots little or no dye is lost during rinsing, in contrast to the bands of the lipoproteins.

#### MEASUREMENT OF DYE UPTAKE BY DIRECT OPTICAL SCANNING

Optical scanning introduces some other errors, which are absent in case of extraction of the dye. Clearing of the paper strips in mineral oil results in solution of dye. Special care is necessary to avoid artifacts by air inclusion. There is a considerable background variation, with consequent difficulty in



selecting a true base line. The most serious drawback is the nonlinearity of the relationship between dye concentration and optical density (91, 26, 54). Finally, with the exception of automatic instruments, optical scanning is more time-consuming than the measurements with elution.

#### CHEMICAL ANALYSIS OF THE LIPOPROTEIN BANDS

We have given full attention to the still imperfect staining methods because of their relative simplicity and the possibility of direct visual qualitative judgment of the results. If these methods are further improved, they will be the methods of choice for the clinical chemist. The much more elaborate methods of chemical analysis of the paper strips allow a differentiation between cholesterol, phospholipids, and neutral fats. Moreover, this kind of analysis may be expected to be more reliable than the staining methods. For research work they have found much appreciation. Especially the cholesterol has attracted a great deal of interest, partly because it is easily estimated in small quantities and partly because it seems most promising in relation with atherosclerosis problems (83, 13, 11, 17, 1, 35, 25, 66). A careful critical study of the cholesterol content of the fractions, obtained by paper electrophoresis was made by Langan, Durrum, and Jencks (66). Micromethods for phospholipids are less practicable and the experience with phospholipids, therefore, is limited.

#### OTHER METHODS OF SEPARATION

Adsorption and the possibility of separation of only small quantities of fractions are handicaps for the paper electrophoresis. Moreover, the procedure results only in the separation of two major groups of substances. Further separation of these alpha and beta groups might be of great importance. The beta lipoproteins are identical with the low-density lipoproteins that may be further separated with ultracentrifugal technics. The question arises whether such separations in the group of alpha or beta lipoproteins may be arrived at with the help of simpler methods, e.g., electrophoresis under other circumstances.

#### STARCH COLUMN ELECTROPHORESIS

It is claimed that the use of starch as a supporting medium instead of filter paper has the advantage of less adsorption. Larger quantities of material may be separated. Many investigators used such columns for more elaborate analytic purposes or for preparative work (64, 65, 121, 36, 41, 42, 1, 28). A very careful study was recently published by the Marburg group (28). Dietrich extracted the various portions of the column in a Soxhlet apparatus during 6 hours with ethanol and then during 1 hour with ether. He recovered about 94 percent of cholesterol, 98 per cent of phospholipids, and 101 per cent of triolein. With the ethanol-ether extraction as described by Kunkel and Slater (65) he obtained recoveries of 70, 78, and 56 per cent, respectively. His



results allow a more detailed study of the quantitative relations between protein, cholesterol, phospholipid, and fatty acids. In his paper, diagrams are shown with his technic, of normal serum and of the serum of a patient with atheromatosis. It is clear that a small alpha-2 fraction is present, the significance of which is little understood as yet. The considerable changes in atheromatosis are obvious. A subdivision within the beta fraction has not yet been succeeded. Kunkel and Slater (64) described a method of electrophoresis in thick layers of filter paper that gave a subdivision within the alpha-1 and beta groups. With fluid film electrophoresis (99, 100) a subdivision in the globulins was reached that may gain importance for lipoprotein subfractionation.

#### FRACTIONATION WITH ETHANOL OR SALTS

Cohn's fractionation technic no. 10 represents another line of approach, based upon other properties than the electrophoretic mobility. It has been specially adapted to the problem of the lipoproteins (24, 70, 104, 89). With this well-known method, two groups of lipoproteins were found with different cholesterol/phospholipid ratio. Lever *et al.* (70) found about 75 per cent of the cholesterol in the group that electrophoretically coincides with the beta globulin. The mean ratio for this group was 2.31, in contrast with a mean of 0.81 for the alpha lipoprotein group. A small-scale adaptation of the first step of Cohn's fractionation method no. 10 was used by Anderson and Keys (3). It is easy enough to be applied in the normal hospital laboratory.

Precipitation of lipoproteins by means of ammonium sulfate was accomplished as early as 1929 (78). Recently, Heydeman (49) made concentration series with sodium sulfate, by adding this salt in portions of 10 Gm./l. serum, up to 300 Gm./l. In the 30 samples, he determined the cholesterol and phospholipids of the supernatant. Between concentrations of 100-240 Gm. sodium sulfate per liter serum, a group of lipoproteins precipitates with a cholesterol/phospholipid ratio of 2.3 (1.7-2.9). A second lipoprotein precipitated at concentrations between 300 and 350 Gm./l. This lipoprotein shows a much lower ratio. For the optimal separation of both groups the author proposes a sodium sulfate concentration of 260 Gm./l. serum. The simplicity of this procedure makes it applicable in routine clinical chemical work. The range, however, found in normal sera is high, moreover, under pathologic conditions, the salting-out properties of the beta lipoproteins may change and erroneous results may be obtained, as for instance in cases of chronic nephritis.

#### COMPARISON OF THE RESULTS OF VARIOUS METHODS

On an average there is a remarkable agreement between the estimation of cholesterol in beta lipoproteins as measured by paper electrophoresis or starch column electrophoresis with subsequent extraction, and with the ethanol or salt-fractionation technics. A synopsis of selected data is given in Table 1.

Table 1. AGREEMENT OF CHOLESTEROL DETERMINATIONS

Method	Group 1	Group 2
Ultracentrifuge (Gofman)	High density	Low density
Electrophoretic mobility	Albumin- $\alpha$ -1-globulin	Beta-globulin
Ethanol fractionation	Stable group	Labile group
Sodium sulfate fractionation	Stable group	Labile group
Cholesterol contents		
With paper electrophoresis	24.6%	75.4%
With starch column electrophoresis	20-30%	58-77%
With ethanol fractionation	25 %	71.4%
With sodium sulfate fractionation	22 %	78 %

## SUBDIVISION WITHIN THE ALPHA AND BETA LIPOPROTEINS

With the Gofman technic, (39), three species of hydrated densities were found in the "high-density lipoproteins" (alpha group). The subdivision in the beta group in Sf values 4-40.000 may represent a large number of species of discrete flotation rates or a continuum between these flotation rate limits. Gofman used for his separation a density of 1.063. One may use other densities too. Havel, Eder, and Bragdon (46) separated a fraction with a density over 1.21 with an electrophoretic mobility of the alpha-1 globulin, practically without cholesterol, but with 10-15 per cent of the serum lipid phosphorus. Separation in the ultracentrifuge was also attempted with use of the density gradient principle of Linderström-Lang, where each substance concentrates in layers with the density of the lipoprotein equal to the density at this place of the sodium chloride solution. This method in combination with Cohn's fractionation permitted the separation and analysis of various atypical lipoproteins (107) in various cases of biliary obstruction, notably in biliary cirrhosis. Three separate abnormal beta lipoproteins have been partially characterized. Further identification may be tried with immunologic methods (70). The increase of the beta lipoproteins in biliary cirrhosis thus proved to consist practically entirely of abnormal lipoprotein (107). Thus, neither electrophoretic mobility nor flotation rate studies give more than a separation into groups of substances.

## CHROMATOGRAPHY OF LIPOPROTEINS

The incidental observation of Carlson (18) that serum lipoproteins are adsorbed onto glass beads gave new possibilities of separation. Preliminary work in this direction proved to be very promising and a separation procedure based upon this property could be developed (19). Serum lipoproteins are adsorbed onto a special glass powder at a pH between 8 and 9. One type of lipoprotein having a cholesterol/phospholipid ratio of 0.3-0.4 is not adsorbed. The alpha and beta lipoproteins may be eluted from the column with buffers

of increasing alkalinity. Alpha and beta groups show various subfractions that may represent various lipoproteins, as may be presumed from the results obtained with sera of patients with atheromatosis, as compared with those of biliary cirrhosis.

#### DYNAMIC STUDIES BY LIPOPROTEIN LIPASE SYSTEMS

Hahn's discovery that an intravenous injection of heparin during a lipemic state produces *in vivo* a clearing of the lactescent serum, has opened a new approach to the studies of the lipoprotein metabolism. From many investigations in this field it became clear that this "clearing factor" may be considered as a lipoproteinase system, involved in the breakdown of the triglyceride moiety of lipoproteins. The enzyme normally occurs intracellularly but appears in the circulation after administration of heparin or of any one of the series of polyanions. During its action, fatty acids and glycerol are liberated (litt. in 43). After heparin injection a sharp decrease of triglycerides (73) and diglycerides was noted, together with a transient sharp increase of the monoglyceride level (20, 21). Moreover, a correlation was found between lipase activity in the serum, and the clearing factor production (93). Addition of pancreatic lipase to lipemic serum shows similar changes in the lipid diagrams, as may be found after addition of clearing factor (132). Albumin acts as a fatty acid acceptor (60). Reaction kinetics are studied (59).

With paper electrophoresis, acceleration of the beta lipoprotein band is noted after a small intravenous injection of heparin. With large doses of heparin, the alpha lipoprotein may run even ahead of the albumin (48, 115). Gordon (43) proved that oleic acid could produce this "lipoprotein-shift" by association with lipoprotein molecules, thus increasing their electrical charge. Transformation of beta into alpha lipoproteins as the reason for the shift is ruled out (48). The protein portions of both show certain differences (16). The general idea is that indeed the lipid portion of the alpha lipoproteins is a product of the breakdown of that of the beta fraction. Never a shift has been found in the direction of the low-density beta lipoproteins (97). Shifts, splitting of bands, or changes in concentration after injection of heparin under various conditions have aroused considerable interest (48, 43, 34, 35, 47, 112, 67, 84, 30, 58, 110, 95, 23, 38, 130).

Practical tests for atheromatosis, whether the clearing effect itself was considered or the "shifting" of the lipoprotein bands (110), have not been obtained as yet. Much work in this field lies ahead, before the problems of the clearing factor have been cleared themselves.

#### LIPOPROTEIN FLOCCULATION TESTS

The discovery that specific pathologic lipoproteins do exist gives new courage to those who hope that a specific flocculation test may be found. Thus the mysticism of flocculation tests finds new adherents, especially in France. The

correlation between beta lipoprotein contents and phenol flocculation, as proposed by Kunkel (62), has been more extensively studied (52, 53, 69, 119). If clearing factor is added to a lipemic serum, the phenol flocculation decreases (94). Another test, with hydrochloric acid, under the name of "Fiche reticulo-endotheliale de Sandor," was proposed (111) and claimed to give typical deviations in its curves (124, 125). As yet, the results are not convincing but the thymol turbidity test of Maclagan is there to prove that a flocculation test may be of great clinical value, notwithstanding the fact that quantitative serum protein and lipoprotein fraction values are provided to the clinic through the strong development of paper electrophoresis. In this field, too, surprises brought about by clinical chemistry are not impossible.

### CLINICAL ASPECTS OF LIPOPROTEIN ANALYSES

The picture of the analytic possibilities makes it clear that, as yet, the information about the lipoproteins in various diseases is largely qualitative, or at best semiquantitative. The quantitative evaluation meets with many controversial results, as may be expected after a critical study of the analytic methods. Moreover, further separation within the groups of lipoproteins is only in its first stage. At the moment, only a rough outline may be given of some of the clinical aspects.

The majority of papers in this field is centered around the problem of atherosclerosis and liver diseases. Other diseases have received less attention, although from those with known disturbances in lipid metabolism some data are available, e.g., in diabetes, lipoid nephrosis (57), myxedema (5), xanthomatosis (81), myelomatosis (109, 74, 108). Various papers gave results in one or more cases of numerous diseases (123, 2, 27, 85, 68, 40) and reviews are already available (63, 118). From these studies a number of interesting data appeared which have in part already been shown and discussed in the introductory part of this paper. A vast field lays ahead for research, which is easily accessible for the clinical chemist. It is impossible, however, to state at this moment, what the clinical significance will be of many of the changes that have been found. A deeper insight into the chemical pathology of these diseases may certainly be expected.

In liver diseases, one of the most constant and obvious changes is the remarkable decrease in the alpha-1 lipoprotein fraction, together with an increase of the beta lipoproteins. The deviations were studied by paper electrophoresis, starch columns, ethanol fractionation, and the Gofman technic (63, 117, 61, 102, 86, 37, 32). Evidence has accumulated that in cases of biliary obstruction and in the early stages of infectious hepatitis (the obstructive stage) plasma lipids are atypically combined with peptides in the form of atypical lipoproteins. In a recent study, Russ, Raymund, and Barr (107) separated three abnormal beta lipoproteins by combining Cohn's fractionation with

density gradient ultracentrifugation. A very important conclusion is that despite similar flotation rates with the Gofman technic, chemical analysis indicates that the lipoproteins in biliary cirrhosis differ from those in atheromatosis.

The problem that has received most attention is that of atheromatosis. Many investigators agree on the fact that in numerous cases in which atheromatosis is clinically proved, or at least highly probable, beta lipoproteins are very high and alpha-1 lipoproteins are low. This applies to cholesterol as well as to the phospholipids and is found with various methods of analysis (6, 7, 4, 105, 44, 119, 131, 113). Also the appearance of a pre-beta band has been noted with paper electrophoresis (27). However, such changes are not always found and they are not typical for atheromatosis. As to the first point, the question arises if atheromatosis may be considered as a continuous biochemical disorder. In medicine we think too often in terms of continuity, but many diseases are in fact paroxysmal derangements. For atheromatosis this view has found much support. The strong physiologic variations in the levels of serum lipids after meals have been considered in this light. As for the second point, the fact is important that the beta lipoprotein changes are especially found in those other diseases that show a higher incidence of secondary atheromatosis (e.g., diabetes, nephrosis, hypometabolism). The separation in alpha and beta lipoproteins as an approach to the problem of atheromatosis seems insufficient. Subdivision within the beta group by means of Gofman's ultracentrifugal technic gives statistically significant differences with "normals," whether concerning the Sf 0-12 group, Sf 12-20 group, or Sf 20-100 group. In an individual case the patterns may be quite normal however.

Many investigators agree that the accumulation of lipids within the walls of the vessels does not exactly reflect the levels or physical state of the blood lipids. Synthesis of cholesterol within the vessel walls may be of importance (134). The significance of other changes in the vessel walls was further stressed by the work of Rinehart, who found disturbances in the metabolism of mucoproteins in the walls to be of major importance.

Moreover, blood coagulability is increased in coronary thrombosis, probably due to an elevation of some lipid components as phosphatidyl ethanolamine and free fatty acids (99, 102). Detailed studies of the fatty acids in serum, by means of chromatographic methods (50), may give new and important information (51).

Intensive further investigations of the whole field will be necessary for a comprehensive picture of the etiology of atheromatosis.

Finally I should like to consider the possibilities of producing changes in disturbed lipoprotein patterns toward normal conditions. Recent observations have shown the normalizing effect of gonadal hormones (106, 88). Cortisone seems to produce similar effects. Blocking of cholesterol resorption from the intestine with help of beta sitosterol gives remarkable effects (8). The effect

of fat or cholesterol restriction in the diet is still subject to much controversy (45). Feeding of unsaturated fatty acids seems to be beneficial (14, 15) and their exclusion from the diet atherogenous (116).

In our laboratory we made an interesting observation in connection with the addition to serum of the macromolecular complex we found in human gallbladder bile (128, 129). The nucleus of this complex consists of molecules of lecithin and desoxycholic acid, bound by the "choleic acid principle." This complex with its molecular weight of about 25,000 is able to bind about 7 molecules of cholesterol per molecule. It may take up further molecules of lecithin to a certain extent. Added to serum, this complex "sweeps away" the total contents of lipids from the lipoproteins, leaving the protein components behind. Bile acids show the same property, giving rise to synthesis of the complex. The acid complex runs ahead of the albumin fraction in paper electrophoresis. It carries a considerable amount of cholesterol in a stable state. Whether it may pass through the vessel walls is uncertain (138). Further study of this remarkable biologic principle of cholesterol stabilization seems gratifying.

### CONCLUSION

I hope this review may succeed in once more convincing the clinical chemist that the general hospital laboratory may be a place for fruitful research work, especially as regards the lipoproteins.

### REFERENCES

1. Ackermann, P., Toro, G., and Kountz, W., *J. Lab. Clin. Med.* **44**, 517 (1954).
2. Adlersberg, D., Bossak, E. T., Sher, I. H., and Sobotka, H., *Clin. Chem.* **1**, 18 (1955).
3. Anderson, J. T., and Keys, A., *Clin. Chem.* **2**, 146 (1956).
4. Antonini, R., Piva, G., Salvinio, L., and Sordi, A., *Giorn. Geront.* **1** (1953).
5. Bansi, H., and Fretwurst, F., *Klin. Wochenschr.* 887 (1954).
6. Barr, D. P., Russ, E. M., and Eder, H. E., *Am. Jour. Med.* **11**, 480 (1951).
7. Barr, D. P., George Brown Memorial Lecture. *Circulation* **8**, 641 (1953).
8. Best, M. M., Duncan, C. H., van Loon, E. J., and Wathen, J. D., *Am. Jour. Med.* **19**, 61 (1955).
9. Biserte, G., Charbonnier, A., and Guerin, F., *Compt. Rend.* **239**, 127 (1954).
10. *Blood Cells and Plasma Proteins*. New York, 1953. (Various contrib. in)
11. Bolinger, R. E., Grady, H. J., and Slinker, B. J., *Am. J. Med. Sc.* **227**, 193 (1954).
12. Boyd, M. F., *J. Biol. Chem.* **101**, 323 (1933), *ibid.* **101**, 61 (1935), **115**, 37 (1936).
13. Boyd, G. S., *Biochem. Jour.* **58**, 680 (1954).
14. Bronte-Stewart, B., et al., *Lancet* **270**, 101 (1956).
15. Bronte-Stewart, B., et al., *Lancet* **270**, 521 (1956).
16. Brown, R. K., Clark, B., Davis, R., and Van Vunakis, H., Comm. No. 6. IIIrd Internat. Conference on lipids. Brussels, July, 1956.
17. Carlson, L. A., *Acta Med. Scand.* 510 (1954).
18. Carlson, L. A., *Acta Chem. Scand.* **9**, 1064 (1955).
19. Carlson, L. A., Comm. No. 7. IIIrd Internat. Conference on lipids. Brussels, July, 1956.
20. Carlson, L. A., and Wadström, L. B., *Clin. chim. acta* **2**, 9 (1957).

21. Carlson, L. A., Wadström, L. B., Comm. No. 8, IIIrd Internat. Conf. on lipids. Brussels, July, 1956.
22. Chargaff, E., *Adv. in Protein Chemistry*, **1**, 1.
23. Christiansen. *Lancet* (1955). Letter to the Editor 11 June 1955.
24. Cohn, E. J., et al., *J. Am. Chem. Soc.* **72**, 465 (1950).
25. Comfort, A., *Biochem. Jour.* **59**, (1955).
26. Crook, E. M., et al., *Biochem. Jour.* **56**, 434 (1954).
27. Dangerfield, W. G., and Smith, E. B., Comm. IIIrd Internat. Congress Biochemistry. Brussels, 1955.
28. Dietrich, F., *Hoppe Seyler's Zeitschr. Physiol. Chem.* **302**, 227 (1955).
29. Dietrich, F., and Jobst, H. (In press.)
30. Dobson, H. L., and Stribling, S., *J. Lab. Clin. Med.* **44**, 788, (1954).
31. Durrum, E., Paul, M., and Smith, E., *Science* **116**, 428 (1952).
32. Eder, H. A., Russ, E. M., Pritchett, R., Wilber, M., and Barr, D. P., *J. Clin. Invest.* **34**, 1147 (1955).
33. Enquete (unpublished) by the Netherlands Society of Clinical Chemistry.
34. Fasoli, A., *Lancet* **262**, 106 (1952).
35. Fasoli, A., *Acta Med. Scand.* **145**, 233 (1953).
36. Flodin, P., and Porath, J., *Biochim & Biophys. Acta* **13**, 175 (1954).
37. Franken, F. H., and Klein, E., *Dtsch. Med. Wschr.* **80**, 1074 (1955).
38. Gerö, S., et al., *Lancet*, No. 6905, Dec. 31, 1955.
39. Gofman, J. W., Glazier, F., Templin, A., Shisower, B., and de Lalla, O., *Physiol. Rev.* **34**, 589 (1954).
40. Gofman, J. W., Rubin, L., McGinley, J., and Jones, H., *Am. J. Med.* **17**, 514 (1954).
41. Grassmann, W., *Angew. Chemie.* **62**, 170 (1950).
42. Grassmann, W., and Hannig, K., *Zeitschr. Physiol. Chemie* **290**, 1 (1952).
43. Gordon, R. S., *J. Clin. Invest.* **34**, 477 (1955).
44. Gottfried, S., Pope, R., Friedman, N., and Dimauro, S., *J. Lab. Clin. Med.* **44**, 651 (1954).
45. Hatch, F. H., Abell, L. L., and Kendall, F. E., *Am. Jour. Med.* **19**, 48 (1955).
46. Havel, R. J., Eder, H. A., and Bragdon, J. H., *J. Clin. Invest.* **34**, 1345 (1955).
47. Herbst, F., and Hurley, N., *J. Clin. Invest.* **33**, 907 (1954).
48. Herbst, F., Lever, W. F., Lyons, M. E., and Hurley, N. A., *J. Clin. Invest.* **34**, 581 (1955).
49. Heydemann, S. F. B., Thesis, Amsterdam, 1953.
50. James, A. T., and Martin, A. J. P., *Biochem. J.* **63**, 144 (1956).
51. James, A. T., and Lovelock, J., Comm. No. 5, IIIrd Internat. Conference on Lipids. Brussels, July, 1956.
52. Jayle, M. F., and Badin, J., *Presse Med.* **61**, 343 (1953).
53. Jayle, M. F., Lagrue, G., and Boussier, G., *Presse Med.* **61**, 1246 (1954).
54. Jencks, W. P., et al., *Biochem. J.* **60**, 205 (1955).
55. Jencks, W. P., and Durrum, E. L., *J. Clin. Invest.* **34**, 1437 (1955).
56. Jones, R. J., Cohen, L., and Corbus, H., *Am. Jour. Med.* **19**, 71 (1955).
57. Klein, E., and Brügel, H., *Zeitsch. Klin. Med.* **153**, 126 (1955).
58. Klein, E., and Franken, F., *Dtsch. Med. Wschr.* **80**, 44 (1955).
59. Korn, E. D., and Quigley, T. W., Comm. No. 23, IIIrd Internat. Conference on Lipids. Brussels, July, 1956.
60. Kubie, G., Comm. No. 26, IIIrd Internat. Conf. on Lipids. Brussels, July, 1956.
61. Küchmeister, H., and Voigt, K., *Dtsch. Arch. Klin. Med.* **201**, 1 (1954).
62. Kunkel, H. G., Ahrens, E., and Eisenmenger, W., *Gastroenterol.* **11**, 499 (1948).
63. Kunkel, H. G., and Ahrens, E., *J. Clin. Invest.* **28**, 1575 (1949).



64. Kunkel, H. G., and Slater, R. J., *J. Clin. Invest.* **31**, 677 (1952).
65. Kunkel, H. G., and Slater, R. J., *Proc. Soc. Exp. Biol. & Med.* **80**, 42 (1952).
66. Langan, T. A., Durrum, E. L., and Jencks, W. P., *J. Clin. Invest.* **34**, 1427 (1955).
67. Laurell, S., *Scand. J. Clin. Lab. Invest.* **7**, 28 (1955).
68. Leinwand, I., *Circulation* **8**, 451 (1953).
69. Lemaire, A., Cottet, J., and Joyeux, R., *Presse Med.* **62**, 1699 (1954).
70. Lever, W. F., et al., *J. Clin. Invest.* **30**, 99 (1951).
71. Levine, L., Kauffman, D. L., and Brown, R. R., *J. Exper. Med.* **102**, 105 (1955).
72. Lindgren, F. T., et al., *J. Phys. & Coll. Chem.* **55**, 80 (1951).
73. Lindgren, F. T., Nichols, A. V., and Freeman, N. K., *J. Phys. Chem.* **59**, 930 (1955).
74. Lewis, L. A., and Page, I. H., *Am. Jour. Med.* **17**, 670 (1954).
75. Man, E., and Gildea, E. F., *J. Biol. Chem.* **122**, 77 (1937), and *J. Clin. Invest.* **15**, 203 (1936).
76. Man, W., *Science* **118**, 86 (1953).
77. Mann, G. V., Munoz, J. A., and Scrimshaw, N. S., *Am. Jour. Med.* **19**, 25 (1955).
78. Macheboeuf, M., *Bull. Soc. Chim. Biol.* **11**, 268 (1929).
79. McDonald, H. J., and Marbach, E. P., Abstr. of papers presented at the 126th meeting of the Am. Chem. Soc., Sept., 1954.
80. McDonald, cit. Jencks and Durrum (55).
81. McGinley, J., Jones, H., and Gofman, J., *J. Invest. Dermatol.* **19**, 71 (1952).
82. Moore, N. S., Ycung, C. M., and Maynard, L. A., *Am. J. Med.* **17**, 348 (1954).
83. Nikkilä, E., *Scand. J. Clin. & Lab. Invest.* **5**, suppl. 8 (1953).
84. Nikkilä, E., and Gräsbeck, R., *Acta Med. Scand.* **150**, 39 (1954).
85. Nys, A., *Rev. Belge Pathol. & Med.* **23**, 329 (1954).
86. Nys, A., *Ud Coll. Lab. Hop. St. Jean. Bruges* 153 (1954).
87. Nys, A., *Chem. Weekblad.* **51**, 643 (1955).
88. Oliver and Boyd, *Circulation* **82** (1956).
89. Oncley, J. L., Gurd, F. R. N., and Melin, M., *J. Am. Chem. Soc.* **72**, 458 (1950).
90. Oncley, J. L., and Gurd, F. R. N., Technical Group, Committee on Lipoproteins and Atherosclerosis. *New York. Acad. Press* 349 (1953).
91. van Os, G. A. J., *Chem. Weekblad.* **49**, 242 (1953).
92. Ott, H., and Roth, W., *Klin. Wochenschr.* **32**, 1099 (1954).
93. Overbeek, G. A., and van der Vies, J., *Biochem. J.* **60**, 665 (1955).
94. Overbeek, G. A., Personal communication.
95. Owen, J. A., *Lancet*, 868, April 23, 1955.
96. Page, I. H., Lewis A. Connor Memorial Lecture. *Circulation* **10**, 1 (1954).
97. Pierce, F. J., Jr., *Metabolism* **3**, 142 (1954).
98. Poole, J. C. F., *Brit. Jour. Exp. Path.* **36**, 248 (1955).
99. Ressler, N., and Jacobson, S. D., *Science* **122**, 1088, (1955).
100. Ressler, N., and Zak, B., *Clin. Chim. Acta* **1**, 392 (1956).
101. Robinson, D. S., and Poole, J. C. F., *Quart. J. Exp. Physiol.* **41**, 36 (1956).
102. Roboz, E., Hess, W., Forster, F., and Temple, D., *Arch. Neurol.* **72**, 154 (1954).
103. Rosenberg, I., *J. Clin. Invest.* **31**, 657 (1952), and *Proc. Soc. Exp. Biol. & Med.* **80**, 751 (1952).
104. Russ, E. M., Eder, H. E., and Barr, D. P., *Am. Jour. Med.* **11**, 468 (1951).
105. Russ, E. M., Eder, H. E., and Barr, D. P., *J. Clin. Invest.* **33**, 1662 (1954).
106. Russ, E. M., Eder, H. E., and Barr, D. P., *Am. Jour. Med.* **19**, 4 (1955).
107. Russ, E. M., Raymund, J., and Barr, D. P., *J. Clin. Invest.* **35**, 133 (1956).
108. Sachs, B., Cady, P., and Ross, G., *Am. Jour. Med.* **17**, 153 (1954).
109. Sachs, B., Cady, P., and Ross, G., *Am. Jour. Med.* **17**, 662 (1954).
110. Sachs, B., and Cady, P., *Circulation* **12**, 503 (1955).



111. Sandor, G., *Bull. Soc. Chim. Biol.* **133**, 1483 (1951).
112. Seanu, A., and Causa, L., *Minerva Med.* **66** (1955).
113. Schettler, G., Dietrich, F., and Eggstein, M., *Verhandl. Dtsch. Gesellsch. Kreislauf. Forschung.* 21ste Tagung. 124 (1955).
114. Smith, E., Crawford, I., Jetton, M., and Durrum, E. L., *Federation Proc.* **12**, 269 (1953).
115. Smith, E. B., and Dangerfield, W. G., Comm. IIIrd Internat. Congress Biochemistry. Brussels, 1955.
116. Sinclair, H. M., Comm. No. 36, IIIrd Internat. Conference on Lipids. Brussels, July, 1956.
117. Snively, J. R., Goldwater, W. H., Randolph, M. L., and Unglaub, W. G., *J. Clin. Invest.* **31**, 664 (1952).
118. Sobotka, H., and Carr, J. J., *Ann. Rev. Med.* **6**, 257 (1955).
119. Soulier, J. P., Alagille, D., and Burstein, M., *Sem. hop. Paris* **29**, 3171 (1953).
120. Spain, D. M., et al., *Am. J. Med. Sc.* **231**, 165 (1956).
121. Svensson, H., and Brattsten, J., *Ark. Kemi* **1**, 401 (1949).
122. Swahn, B., *Scand. J. Clin. & Lab. Invest.* **4**, 98 (1952).
123. Swahn, B., *Scand. J. Clin. & Lab. Invest.* **5**, suppl. 9 (1953).
124. Vargues, R., *Ann. Inst. Pasteur.* **84**, 466 (1953).
125. Vargues, R., et al., *Compt. Rend. Soc. Biol.* **147**, 112 (1953).
126. Verschure, J. C. M., and Hoefsmit, I., *Ned. Tydschr. Geneesk.* **98**, 3410 (1954).
127. Verschure, J. C. M. Unpublished data.
128. Verschure, J. C. M., *Clin. Chim. Acta* **1**, 38 (1956).
129. Verschure, J. C. M., and Mijnlief, P. F., *Clin. Chim. Acta* **1**, 154 (1956).
130. Visser, J. Letter to the Editor. *Lancet* April 21, 1956.
131. Voigt, K., and Schrader, E., *Zeitschr. Kreislaufforschung.* **43**, 2 (1954).
132. Weld, G. B., Comm. IIIrd Internat. Conference on Lipids. Brussels, July 1956.
133. Werthessen, N. T., and Nyman, M. A., Comm. No. 37, IIIrd Internat. Conf. Lipids. Brussels, July 1956.
134. Litt in Wuhrmann, F., and Wunderly, C., *Die Bluteiweisskörper des Menschen.* Basel, 1952.
135. Wunderly, C., and Pezold, F., *Naturwiss* **39**, 493 (1952).
136. Wunderly, C., *Die Papierelektrophorese.* Aarau, 1954.
137. Wunderly, C., and Wieme, R. J., *Arch. Int. Physiol. Biochem.* **63**, 318 (1955).
138. Byers, S. O., *J. Exp. Med.* **97**, 511 (1953).

## Members of The 1956 International Congress of Clinical Chemistry

**A**PPROXIMATELY 700 CLINICAL CHEMISTS, representing 34 countries, attended the International Congress of Clinical Chemistry held at the Barbizon-Plaza Hotel, New York City, September 9-14, 1956. The Congress was held under the auspices of the American Association of Clinical Chemists, by authorization of the International Federation of Clinical Chemistry and the Commission on Clinical Chemistry of the International Union of Pure and Applied Chemistry.

Through the good offices of the National Science Foundation and fourteen members of American chemical industry, the American Association of Clinical Chemists was able to invite and partially subsidize the travel expenses for thirty foreign scientists. Many of the foreign scientists participated in five symposia which formed the nucleus for 17 sessions of contributed papers. Besides the invited guests were the official representatives of the various foreign scientific societies and the representatives of both foreign and United States governmental agencies.

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 Bowman, Russel O., *Denver, Col.*  
 Boy, G., *Reims, France*  
 Boyd, Harriet M., *Philadelphia, Pa.*  
 Bral, M., *Teheran, Iran*  
 Brewer, William, *Oakdale, N. J.*  
 Briese, S. F., *Minneapolis, Minn.*  
 Briggs, Anglis R., *Wilmington, Del.*  
 Brogna, Donato, *Brooklyn, N. Y.*  
 Brot, Nathan, *Brooklyn, N. Y.*  
 Brown, Harold H., *Pawtucket, R. I.*  
 Brown, Virginia C., *Lancaster, Pa.*  
 Brown, William R., *Sayre, Pa.*  
 Brun, Claus, *Copenhagen, Denmark*  
 Bruns, F. H., *Dusseldorf, Germany*  
 Bunch, Leitha D., *Winifred, Kan.*  
 Burch, James F., *Greensboro, N. C.*  
 Burger, Martin, *Brooklyn, N. Y.*  
 Bryon, Charles S., *Brooklyn, N. Y.*

## C

Cabaud, Philip G., *Brooklyn, N. Y.*  
 Calderon, M. Theresa, *Mexico, D. F.*  
 Cammisia, Guido F., *Alexandria, Va.*  
 Campbell, Edna Andrews, *Detroit, Mich.*  
 Campbell, Frank M., *Sharon, Conn.*  
 Cancio, Marta, *San Juan, P. R.*  
 Caraway, Mrs., *Providence, R. I.*  
 Caraway, Wendell T., *Providence, R. I.*  
 Carlson, Arthur, *Glen Cove, N. Y.*  
 Carmichael, Emmett B., *Birmingham, Ala.*  
 Carr, Julius, *New York, N. Y.*  
 Cecalas, Connie, *Upper Darby, Pa.*  
 Charles, Eleanor, *Spring Valley, N. Y.*  
 Chediak, M., *Havana, Cuba*  
 Cherniak, Joseph M., *Flushing, N. Y.*  
 Chilcote, Max E., *Buffalo, N. Y.*  
 Clark, Roberta, *St. John, N. B.*  
 Clifton, Eugene E., *Ithaca, N. Y.*  
 Cohen, Murray, *New York, N. Y.*  
 Cohen, Sidney, *Boston, Mass.*  
 Cohn, David J., *Park Forest, Ill.*  
 Collins, Margaret C., *Philadelphia, Pa.*  
 Connolly, Valentine J., *Staten Island, N. Y.*  
 Connor, Thomas H., *Providence, R. I.*  
 Consolazio, William V., *Washington, D. C.*

Cooper, Gerald R., *Chamblee, Ga.*  
 Cornatzer, W. E., *Grand Forks, N. D.*  
 Cornelius, L., *New York, N. Y.*  
 Coulombe, J. J., *Sherbrooke, P. Q., Can.*  
 Coursin, D. B., *Lancaster, Pa.*  
 Craig, John Cymeran, *Oxford, England*

## D

Daily, Norman H., *Painesville, Ohio*  
 Davies, D. A. L., *Salisbury, England*  
 Davis, Robert J., *Tampa, Fla.*  
 Davis, Robert L., *Baltimore, Md.*  
 Davis, Sidney, *Indianapolis, Ind.*  
 Deane, Burton C., *Brooklyn, N. Y.*  
 Dempsey, Mary E., *Minneapolis, Minn.*  
 Deutsch, Marshal E., *Morris Plains, N. J.*  
 Dickerson, Henry, *Bethesda, Md.*  
 Dickman, Albert, *Philadelphia, Pa.*  
 Di Ferrante, Nicola, *Upton, N. Y.*  
 DiGiacomo, Madelina, *New York, N. Y.*  
 Donovan, Paul K., *Brooklyn, N. Y.*  
 Dotti, Louis B., *New York, N. Y.*  
 Dreisbach, Lorraine, *Philadelphia, Pa.*  
 Dryer, Robert L., *Iowa City, Iowa*  
 Dryfoos, Herbert L., *San Francisco, Calif.*  
 Duane, Regina, *Allston, Mass.*  
 Dubowski, Kurt M., *Des Moines, Iowa*  
 Duggan, Daniel E., *Bethesda, Md.*  
 Dunaway, Mary, *Jackson, Miss.*  
 Durrum, E. L., *Stanford, Calif.*  
 Dworecki, Izaak J., *New York, N. Y.*

## E

Edgar, J. V. D., *Manhasset, N. Y.*  
 Eichel, Bertram, *Brooklyn, N. Y.*  
 Eichen, Seymour, *Allentown, Pa.*  
 Eicher, Maynard, *Bethesda, Md.*  
 Eleet, Bernice L., *St. Paul, Minn.*  
 Elkan, Bruno, *Brooklyn, N. Y.*  
 Elkinton, J. Russel, *Philadelphia, Pa.*  
 Ellerbrook, Lester D., *Seattle, Wash.*  
 Elliott, Howard C., *Birmingham, Ala.*  
 Erk, Vernon Otto, *New York, N. Y.*  
 Escobar, Ysabel A., *Mexico, D. F.*  
 Ettman, Samuel L., *Miami, Fla.*  
 Eusebi, Albert, *Youngstown, Ohio*

## F

Fales, Frank W., *Emory, Ga.*  
 Fasken, J. E., *Toronto, Ontario, Can.*  
 Febbaro, Elsie, *Union City, N. J.*  
 Federico, Olga, *New York, N. Y.*  
 Feldstein, Milton, *Buffalo, N. Y.*  
 Fenichel, Richard L., *Detroit, Mich.*  
 Ferguson, Marion H., *Winnipeg, Can.*  
 Ferrari, Andres, *Chauncey, N. Y.*

Fine, Charles, *Philadelphia, Pa.*  
 Fiorese, Francesco, *New York, N. Y.*  
 Fitzgerald, John T., *Portland, Maine*  
 Fitzpatrick, William H., *Alexandria, Va.*  
 Flanagan, T. L., *Philadelphia, Pa.*  
 Flynn, John E., *New York, N. Y.*  
 Fogerty, Eleanor G., *Watertown, Mass.*  
 Foster, J. B. T., *New York, N. Y.*  
 Fox, Charles L., *New York, N. Y.*  
 Fraenkel-Conrat, Jane, *Oakland, Calif.*  
 Frame, Elizabeth G., *Bethesda, Md.*  
 Frankel, Sam, *St. Louis, Mo.*  
 Franklin, Edward C., *New York, N. Y.*  
 Free, Alfred H., *Elkhart, Ind.*  
 Free, Helen M., *Elkhart, Ind.*  
 Freeman, Monroe E., *New York, N. Y.*  
 Friedman, Howard S., *New Mexico*  
 Friedman, Max, *New York, N. Y.*  
 Fromals, J., *Cachan (Seine), France*

## G

Gambino, S. Raymond, *Milwaukee, Wis.*  
 Gates, Elaine, *Augusta, Ga.*  
 Gawkowski, Albert S., *Jamaica, N. Y.*  
 Genest, Jacques, *Montreal, Can.*  
 Gerarde, H. W., *Westfield, N. J.*  
 Gershenfeld, Lester H., *New York, N. Y.*  
 Gilman, Mrs. L., *Lake Hopatcong, N. J.*  
 Giovannelli, Thomas, *Boston, Mass.*  
 Gittleman, Isaac, *Brooklyn, N. Y.*  
 Glass, George, *New York, N. Y.*  
 Glauberman, Rachel, *Brooklyn, N. Y.*  
 Goeckel, Henry, *Cranford, N. J.*  
 Goldberg, Aaron, *New York, N. Y.*  
 Goldberg, C. A. J., *Philadelphia, Pa.*  
 Golden, Walter R. C., *Stanford, Conn.*  
 Goldenberg, Harry, *Glen Oaks, N. Y.*  
 Goldschmidt, Leontine, *Queens Village, N. Y.*  
 Golub, Murray, *Oswego, N. Y.*  
 Gomes da Costa, Silverio Ferrera, *Lisbon, Portugal*  
 Gorman, Frank, *Farmingdale, N. Y.*  
 Gottfried, Sidney, *Bridgeport, Conn.*  
 Gottschall, G. Y., *New York, N. Y.*  
 Gouge, Susan C. J., *Washington, D. C.*  
 Gray, Edward W., *West Brentwood, N. Y.*  
 Gray, Irving, *Washington, D. C.*  
 Grayzel, Harold G., *Brooklyn, N. Y.*  
 Greenberg, J. R., *San Francisco, Calif.*  
 Greenblatt, I. J., *Brooklyn, N. Y.*  
 Greenspan, Ezra M., *Brooklyn, N. Y.*  
 Greenstein, Alex I., *New York, N. Y.*  
 Grelis, Mary E., *Bloomfield, N. J.*  
 Gross, Milton, *Jersey City, N. J.*  
 Grunbaum, Benjamin W., *Minneapolis, Minn.*  
 Gubernick, Issadore, *Jamaica, N. Y.*

Guillot, Marcel H., *Paris, France*  
 Gurd, Frank R. N., *New York, N. Y.*

## H

Habaush, Edward J., *New York, N. Y.*  
 Hadley, Susan J., *New York, N. Y.*  
 Haidar, George Abu, *Beirut, Lebanon*  
 Hainline, Adrian, *Cleveland, Ohio*  
 Hald, Pauline M., *New Haven, Conn.*  
 Hall, H. Phoebe, *New York, N. Y.*  
 Hamburger, Jean, *Paris, France*  
 Hamilton, Paul B., *Wilmington, Del.*  
 Hanok, Albert, *Bronx, N. Y.*  
 Harrison, Esther, *Philadelphia, Pa.*  
 Harrison, J. W. E., *Philadelphia, Pa.*  
 Harwell, Bryan, *Shreveport, La.*  
 Harvey, T. S., *Princeton, N. J.*  
 Heftmann, Erich, *Bethesda, Md.*  
 Henley, Alfred, *Riverdale, Md.*  
 Hernandez, Aurelio, *Havana, Cuba*  
 Herrate, Enrique, *Guatemala City, Guatemala*  
 Hill, Robert M., *Denver, Col.*  
 Hilton, James G., *New York, N. Y.*  
 Hinsberg, Karl, *Dusseldorf, Germany*  
 Hirose, Ruby, *Dayton, Ohio*  
 Hjelt, Inga, *New York, N. Y.*  
 Hogan, Ralph, *Chamblee, Ga.*  
 Hordynsky, Walter, *Newark, N. J.*  
 Horejst, Jaroslav, *Prague, Czech.*  
 Horowitz, Myer G., *Cincinnati, Ohio*  
 Horti, Michael, *Richmond Hill, N. Y.*  
 Hamilton, R. H., *Philadelphia, Pa.*  
 Hudson, Leona, *Buffalo, N. Y.*  
 Huisman, T. H. J., *Groningen, Netherlands*  
 Hunt, Marian, *Madison, Wis.*

## I

Ilka, Stephanie J., *New York, N. Y.*  
 Irish, Oliver, J., *Washington, D. C.*  
 Isenberg, Henry D., *Long Island, N. Y.*

## J

Jackson, Sanford H., *Toronto, Ontario, Can.*  
 Jacobssohn, Gert M., *Lafayette, Ind.*  
 Jablons, Benjamin, *New York, N. Y.*  
 Jablonski, Ernest S., *Buffalo, N. Y.*  
 Jaime, Aida, *Havana, Cuba*  
 Jakab, Gizella, *Mt. Vernon, N. Y.*  
 Jameson, Dorothy, *Coatesville, Pa.*  
 Jenkins, Dal G., *Rahway, N. J.*  
 Jijon, Miguel, *Ecuador*  
 Johnson, B. Connor, *Urbana, Ill.*  
 Johnson, David F., *Bethesda, Md.*  
 Jones, Mildred, *Philadelphia, Pa.*

Jonnard, Raymond, *Paterson, N. J.*  
 Jossens, J. V., *Louvain, Belgium*  
 Josephson, Bertil, *Stockholm, Sweden*

## K

Kachmar, John F., *Philadelphia, Pa.*  
 Kahn, Samuel G., *New Brunswick, N. J.*  
 Kanter, Saul L., *Palo Alto, Calif.*  
 Kaser, Margaret M., *Wood, Wis.*  
 Keegan, Patricia K., *Philadelphia, Pa.*  
 Kegeles, Gerson, *Worcester, Mass.*  
 Keller, Charles W., *Rutherford, N. J.*  
 Kelly, Jacques M., *New Brunswick, N. J.*  
 Kemnitzer, Arthur G., *Albany, N. Y.*  
 Kessler, David, *Brooklyn, N. Y.*  
 Kessler, Gerald, *Philadelphia, Pa.*  
 Kibrick, Andre C., *New York, N. Y.*  
 King, Earl Judson, *London, England*  
 Kirby, John K., *Austin, Texas*  
 Kirman, David, *East Orange, N. J.*  
 Klein, Bernard, *Bronx, N. Y.*  
 Klein, Irving F., *Staten Island, N. Y.*  
 Kleiner, Israel S., *New York, N. Y.*  
 Kleschick, Agnes F., *Philadelphia, Pa.*  
 Klugerman, Maxwell B., *Baltimore, Md.*  
 Knowlton, Marjorie, *Washington, D. C.*  
 Koch, Paul, *Montreal, Can.*  
 Koehler, L. H., *Bethlehem, Pa.*  
 Kosinski, Alexander, *Johnston City, N. Y.*  
 Koster, Henry F., *New York, N. Y.*  
 Kotake, Yahito, *Wakayama City, Japan*  
 Kozelka, Frank L., *Madison, Wis.*  
 Kramer, Hildegard, *St. Louis, Mo.*  
 Kream, Jacob, *New York, N. Y.*  
 Ku, Jung Yu, *China*  
 Kunkel, Henry G., *New York, N. Y.*  
 Kyker, Granvil C., *Oak Ridge, Tenn.*

## L

Lanchan, G. F., *Ft. Sam Houston, Texas*  
 Lapan, Bernard, *New York, N. Y.*  
 Lapointe, Marcell, *Montreal, Can.*  
 La Rocca, Rudolph, *Hoboken, N. J.*  
 Larson, Corrine A., *Baltimore, Md.*  
 Lasker, M., *Yonkers, N. Y.*  
 Laufer, Annie, *New York, N. Y.*  
 Lavine, Leroy S., *New Hyde Park, N. Y.*  
 Law, N. C., *Ottawa, Ontario, Can.*  
 Lawrence, Grace, *Chicago, Ill.*  
 Lawrie, Hubert, *Lancashire, England*  
 Layton, William M., *Pearl River, N. Y.*  
 Leeper, Robert D., *Brooklyn, N. Y.*  
 Lehmann, H., *London, England*  
 Leiboff, Samuel, *Long Branch, N. J.*  
 Leifheit, Howard C., *Washington, D. C.*  
 Leinward, Irving, *New York, N. Y.*

Leitner, M. J., *E. Stroudsburg, Pa.*  
 Lemus, Concha, *El Salvador, C. A.*  
 Leonard, Reid, *Pensacola, Fla.*  
 Levin, Robert A., *Norwich, N. Y.*  
 Levine, Victor E., *Omaha, Neb.*  
 Lewis, George T., *Coral Gables, Fla.*  
 Lewis, Paul L., *Philadelphia, Pa.*  
 Lichwell, Helen, *West Roxbury, Mass.*  
 Lippard, Dickinson, *Harrisburg, Pa.*  
 Lochmead, H. B., *Woodbury, N. J.*  
 Lo Cricchio, John, *Youngstown, Ohio*  
 London, Morris, *Flushing, N. Y.*  
 Long, William, *Philadelphia, Pa.*  
 Lucuta, Evangeline E., *Windsor, Ont., Can.*  
 Lugouoy, Julius K., *Bronx, N. Y.*  
 Lulias, Sultana T., *Philadelphia, Pa.*

## M

MacIntyre, Iain, *London, England*  
 MacLagan, Noel F., *London, England*  
 Maher, John R., *Denver, Colo.*  
 Malament, Sylvia, *Morristown, N. J.*  
 Man, Evelyn, *New Haven, Conn.*  
 Mandel, Emanuel E., *Chicago, Ill.*  
 Mangum, George H., *Morris Plains, N. J.*  
 Manon del Rio, J. R., *Dominican Republic*  
 Margaria, R., *Milan, Italy*  
 Maria Consolata, Sister, *Columbus, Ohio*  
 Marino, Jean, *Philadelphia, Pa.*  
 Marius, Nathan, *Brooklyn, N. Y.*  
 Marsh, Walton H., *Brooklyn, N. Y.*  
 Marshak, Celia L., *Woods Hole, Mass.*  
 Martin, Marcus, *Bronx, N. Y.*  
 Maruyama, George M., *Dubuque, Iowa*  
 Mason, Morton F., *Dallas, Texas*  
 Mason, W. B., *Rochester, N. Y.*  
 Mass, Morris, *Kew Gardens, L. I., N. Y.*  
 Matallana, Alfonso, *Calí, Colombia*  
 Maurukas, F., *Elyria, Ohio*  
 Marvelis, William P., *Gerda*  
 Mayer, Gerda G., *New York, N. Y.*  
 Mazarella, Nicholas M., *New York, N. Y.*  
 McCann, Barbara, *Madison, Wis.*  
 McCoord, Augusta B., *Rochester, N. Y.*  
 McDonald, Hugh, *Chicago, Ill.*  
 McGeown, Mary G., *Belfast, N. Ireland*  
 McKenna, Mary Helen, *New York, N. Y.*  
 McLaughlin, Joseph, *Washington, D. C.*  
 McMenemy, Rapier, *Boston, Mass.*  
 McNair, Ruth Davis, *Detroit, Mich.*  
 Meites, Samuel, *Columbus, Ohio*  
 Menne, F., *Germany*  
 Mershon, Jeanne, *Bloomfield, N. J.*  
 Meshaka, Souheil, *Damas, Place Merje*  
 Miller, Elizabeth E., *Cochituate, Mass.*  
 Miller, Myer, *Middletown, N. Y.*

Molnar, Nicholas M., *New York, N. Y.*  
 Moore, Stanford, *New York, N. Y.*  
 Moran, John J., *Philadelphia, Pa.*  
 Moreira, Oramar, *Brasil*  
 Moreland, Ferrin B., *Houston, Texas*  
 Morgenstern, Stanley, *Brooklyn, N. Y.*  
 Morrison, D. B., *Memphis, Tenn.*  
 Mulvihill, John G., *Brooklyn, N. Y.*  
 Murphy, James P., *Dayton, Ohio*  
 Murray, Edward J., *Newark, N. J.*  
 Murray, Martin H., *Rochester, N. Y.*  
 Myers, Patricia J., *Milton, Mass.*

## N

Napier, Edward A., *Pittsburgh, Pa.*  
 Natelson, Samuel, *Rockford, Ill.*  
 Neher, Robert, *Basle, Switzerland*  
 Nelson, Margaret S., *Long Island, N. Y.*  
 Nemshick, Mary L., *Jersey City, N. J.*  
 Ness, Arthur T., *Bethesda, Md.*  
 Neufeld, A. H., *Montreal, Can.*  
 Newman, Clinton J., *Wildwood, N. J.*  
 Newmark, Harold, *New York, N. Y.*  
 Nishi, Hiroshi, *Bethesda, Md.*  
 Nobel, Sidney, *Long Branch, N. J.*  
 Nordmann, Jo, *Paris, France*  
 Nordmann, Roger, *Paris, France*  
 Novick, William J., *Elkins Park, Pa.*  
 Nowaczynski, W., *Montreal, Can.*  
 Nuss, Daisy, *Philadelphia, Pa.*

## O

O'Hagan, John E., *Queensland, Australia*  
 Ohshima, Kenzo, *Tokyo, Japan*  
 Oldham, Ellis C., *Des Moines, Iowa*  
 Oreskes, Irwin, *Brooklyn, N. Y.*  
 Orozco, Fernando Velez, *Mexico, D. F.*  
 Oser, Bernard L., *Long Island City, N. Y.*  
 Osgood, Bess G., *South Bend, Ind.*  
 Ostenberg, A. T., *Chicago, Ill.*  
 O'Sullivan, Michael, *Toronto, Ontario, Can.*  
 O'Toole, James J., *Iowa City, Iowa*  
 Owen, John A., *Edinburgh, Scotland*

## P

Packman, Elias W., *Philadelphia, Pa.*  
 Papadopolou, Daphne, *Bethesda, Md.*  
 Pasternack, Josephine, *Philadelphia, Pa.*  
 Patterson, E. B., *Philadelphia, Pa.*  
 Paubionsky, Peace, *Philadelphia, Pa.*  
 Payne, Mary Ann, *New York, N. Y.*  
 Peeters, Hubert, *Belgium*  
 Perkins, Ella, *Lansdowne, Pa.*  
 Perri, Giulio C., *Douglasston, N. Y.*  
 Petermann, Mary L., *New York, N. Y.*

Peters, Theodore, *Cooperstown, N. Y.*  
 Peterson, Ralph E., *Bethesda, Md.*  
 Pineus, Joseph B., *Brooklyn, N. Y.*  
 Pitner, Georgia, *West Roxbury, Mass.*  
 Pollack, Evelyn, *Newark, N. J.*  
 Pomerene, Elizabeth, *Cleveland, Ohio*  
 Porter, D. L., *New York, N. Y.*  
 Power, M. H., *Rochester, Minn.*  
 Preshmore, M. L., *West Point, Pa.*  
 Prestrud, Mildred, *Kalamazoo, Mich.*  
 Prince, John D., *Hollywood, Calif.*  
 Princiotto, J. V., *Washington, D. C.*  
 Pringle, B. H., *Dearborn, Mich.*  
 Prytz, B. O., *Roslyn, N. Y.*  
 Pugliese, Ralph J., *New Brunswick, N. J.*  
 Purcell, May K., *Philadelphia, Pa.*

## R

Rakowski, Frances E., *Sayre, Pa.*  
 Rano, Royden N., *Rochester, N. Y.*  
 Ransom, Charles G., *Nashville, Tenn.*  
 Ratner, Harold, *Brooklyn, N. Y.*  
 Raymunt, Julia, *New York City, N. Y.*  
 Reiner, Miriam, *Washington, D. C.*  
 Reinhold, John G., *Philadelphia, Pa.*  
 Rice, Eugene W., *Pittsburgh, Pa.*  
 Rice, Vincent E., *Erie, Pa.*  
 Rich, Clayton, *New York, N. Y.*  
 Richardson, Harriet K., *Philadelphia, Pa.*  
 Richeimer, Ralph, *New York, N. Y.*  
 Richter, Helmut, *Ottawa, Ontario, Can.*  
 Riegel, Cecilia, *Philadelphia, Pa.*  
 Rivera, Jose A., *San Antonio, Texas*  
 Roberts, Arlan G., *Chicago, Ill.*  
 Robinson, H. W., *Philadelphia, Pa.*  
 Roboz, Elizabeth, *Washington, D. C.*  
 Roe, Joseph H., *Washington, D. C.*  
 Romanovich, Helen, *Peckville, Pa.*  
 Rosbach, David O., *Roanoke, Va.*  
 Rosenberg, Arthur A., *Albany, N. Y.*  
 Rosenberg, Bernard, *Stamford, Conn.*  
 Rosenthal, Harold L., *Rochester, N. Y.*  
 Routh, Joseph I., *Iowa City, Iowa*  
 Rubin, Martin, *Washington, D. C.*  
 Rundell, Anna L., *Coral Gables, Fla.*  
 Russ, Ella M., *New York, N. Y.*  
 Ryland, Margaret E., *Philadelphia, Pa.*

## S

Sadler, John, *St. Petersburg, Fla.*  
 Saifer, Abraham, *Brooklyn, N. Y.*  
 Sall, Theodore, *Philadelphia, Pa.*  
 Salmon, A. Austin, *New York, N. Y.*  
 Salzberg, Gertrude, *Brooklyn, N. Y.*  
 Samachson, Joseph, *New York, N. Y.*  
 Sammons, H. G., *Birmingham, England*

Sample, Albert B., *Merion, Pa.*  
 Samson, Meyer, *Philadelphia, Pa.*  
 Samuelsen, George S., *Brooklyn, N. Y.*  
 Sang, James B., *New York, N. Y.*  
 Sanz, M., *Geneva, Switzerland*  
 Saunders, Joseph F., *Washington, D. C.*  
 Saunders, Rex A., *Birmingham, England*  
 Searlata, Thomas, *Marlborough, Mass.*  
 Schachter, Yette, *New York, N. Y.*  
 Schaffer, Karl, *Roselle, N. J.*  
 Schelling, Victor, *Detroit, Mich.*  
 Schloss, Martin F., *Eacine, Wis.*  
 Schmerzler, Emanuel, *Brooklyn, N. Y.*  
 Schmidt, Marilyn R., *Milltown, N. J.*  
 Schnack, Herbert, *Vienna, Austria*  
 Schoolman, Joseph, *New York, N. Y.*  
 Schucher, Reuben, *Montreal, Can.*  
 Schultz, Eugene L., *Jackson Heights, N. Y.*  
 Schutte, Julio F., *Havana, Cuba*  
 Schwartz, Morton A., *New York, N. Y.*  
 Schwartz, Morton K., *New Hyde Park, N. Y.*  
 Schwartz, Samuel, *Minneapolis, Minn.*  
 Schwarz, Henry P., *Philadelphia, Pa.*  
 Seligson, David, *Philadelphia, Pa.*  
 Selvey, Keith, *San Francisco, Calif.*  
 Sendroy, Julius, *Bethesda, Md.*  
 Senesky, Dorothy J., *Philadelphia, Pa.*  
 Senior, Kenneth L., *Philadelphia, Pa.*  
 Sherber, Daniel A., *Bronx, N. Y.*  
 Sherman, Burton S., *Brooklyn, N. Y.*  
 Shetlar, M. R., *Oklahoma City, Okla.*  
 Shetlar, Clara L., *Oklahoma City, Okla.*  
 Schwachman, Harry, *Boston, Mass.*  
 Siegel, Malcom, *New York, N. Y.*  
 Silber, Robert H., *Rahway, N. J.*  
 Silhavy, Ernest, *New Britain, Conn.*  
 Siliprandi, Noris, *Rome, Italy*  
 Silver, H., *New York, N. Y.*  
 Simmons, Norman W., *Toronto, Can.*  
 Sindram, E. D. A., *Curacao, N.W.I.*  
 Skeggs, Leonard T., *Cleveland, Ohio*  
 Sloan, Charles H., *Ann Arbor, Mich.*  
 Slocum, Jewell Rebecca, *Jackson, Miss.*  
 Slonim, Arnold R., *Lynn, Mass.*  
 Small, Carrol S., *Loma Linda, Calif.*  
 Smeby, Robert R., *Elkhart, Ind.*  
 Smeets, William, *Eindhoven, Netherlands*  
 Smith, Hinton C., *Holbrook, Mass.*  
 Smith, Margaret E., *Auburn, N. Y.*  
 Sobel, Albert E., *Brooklyn, N. Y.*  
 Sobotka, Harry, *New York, N. Y.*  
 Solow, Robert B., *Indianapolis, Ind.*  
 Sommermeyer, Viola, *San Diego, Calif.*  
 Soupard, Pierre, *Brussels, Belgium*  
 Sperry, Warren M., *New York, N. Y.*  
 Sprince, Herbert, *Coatesville, Pa.*  
 Stary, Z., *Istanbul, Turkey*

Steelman, Elaine, *Atlantic City, N. J.*  
 Stern, Joel R., *Chicago, Ill.*  
 Stern, Milton, *Boston, Mass.*  
 Sternberg, Joseph, *Montreal, Can.*  
 Stewart, C. P., *Edinburgh, Scotland*  
 Stires, Wayne, *Columbus, Ohio*  
 Stockton, C. B., *Bakersfield, Calif.*  
 Stone, Gilbert, *New York, N. Y.*  
 Stoudt, John M., *Cincinnati, Ohio*  
 Straw, Richard F., *Wichita, Kan.*  
 Strickman, Robert L., *Bayside, N. Y.*  
 Stueck, George H., *White River Junction, Vt.*  
 Stull, Arthur, *Washington, D. C.*  
 Sunderman, William, *Philadelphia, Pa.*  
 Sunderman, William, Jr., *Philadelphia, Pa.*  
 Sunshine, Irving, *Cleveland, Ohio*

## T

Tabachnick, Elizabeth, *New York, N. Y.*  
 Tabenkin, Philip A., *Peekskill, N. Y.*  
 Takerhara, Kenneth N., *Johnstown, Penna.*  
 Tanino, Jun, *Brooklyn, N. Y.*  
 Tannenbaum, H. S., *Bath, N. Y.*  
 Taussky, H. H., *New York, N. Y.*  
 Taylor, Haywood M., *Durham, N. C.*  
 Taylor, Richard C., *Philadelphia, Pa.*  
 Tietz, Norbert W., *Richmond, Ind.*  
 Tonks, D. B., *Ottawa, Ontario, Can.*  
 Tria, Eusebio, *Ferrara, Italy.*  
 Tsenghi, Christina, *New York, N. Y.*  
 Tuller, Elizabeth, F., *Boston, Mass.*  
 Turner, D. A., *Washington, D. C.*

## V

Vanderau, Margaret, *Philadelphia, Pa.*  
 van der Schaaf, P. C., *Curacao, N.W.I.*  
 Van Slyke, D. D., *Upton, N. Y.*  
 Vasta, Bruno, *Bethesda, Md.*  
 Vaughn, Ruth D., *Brooklyn, N. Y.*  
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